Influence of human activity on gut microbiota and immune responses of Darwin's finches in the Galápagos Islands

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ABSTRACT

Urbanization can influence animal traits, including immunity and gut microbiota. Over the past several decades, the Galápagos Islands have seen rapid resident human population growth and tourist activity, leading to varying levels of human activity across Islands. Consequently, diet, gut microbiota, and immunity of endemic animals, such as Darwin's finches, may have changed. The goal of this study was to determine the effect of land use on the immune response, gut microbiota, and body measurements of Darwin's finches in 2008, at a time of rapidly increasing human activity in the Islands. Specifically, we compared proxies of immunity (lysozyme activity, and haptoglobin, complement antibody, and natural antibody levels), gut microbiota (bacterial diversity, community structure and membership, and relative abundance of bacterial taxa), and body measurements (body mass, tarsus length, and scaled mass index) across undeveloped, agricultural, and urban areas for medium ground finches (*Geospiza fortis*) and small ground finches (*G. fuliginosa*). We found that lysozyme activity was lower and observed bacterial species richness was higher in urban areas compared to non-urban areas across both finch species. For small ground finches, relative abundances of three bacterial genera (*Pseudoxanthomonas*, *Cloacibacterium*, and *Dietzia spp.*) were higher in urban areas compared to non-urban areas, but this pattern was not observed in medium ground finches. Medium ground finches were smaller in undeveloped areas compared to the other two areas, but body measurements of small ground finches did not differ across areas. Our results suggest that human activity can impact immune measures and gut microbiota of Darwin's finches.

INTRODUCTION

Human activity is rapidly changing the natural environment, which can influence avian ecology and evolution. For example, anthropogenic change can influence many avian traits, including morphology (e.g., bill size), behavior (e.g., boldness, predator avoidance, problem solving), and physiology (e.g., digestion, immunity) (Hendry *et al.*, 2006; Møller, 2008; Atwell *et al.*, 2012; Samia *et al.*, 2017; Sol *et al.*, 2018; Strandin *et al.*, 2018). One factor that can mediate the effect of human activity on avian traits is changes in food availability. Humans can change food resource availability for birds by increasing the abundance of unnatural food through wild bird feeders, the disposal of human food waste into the environment, and by reducing the abundance of natural food items through environment changes (Murray *et al.*, 2016; Bosse *et al.*, 2017; Start *et al.*, 2018). In turn, birds lacking natural food resources may be forced to invest less in maintaining body mass or physiological processes, such as immune responses, which can be energetically costly (Sheldon and Verhulst, 1996; Svensson *et al.*, 1998; Lochmiller and Deerenberg, 2000; Demas, 2004; Sternberg *et al.*, 2012; Cornet *et al.*, 2014; Howick and Lazzaro, 2014).

In addition, human activity can alter avian traits through impacts on the gut microbiota, the community of symbiotic microbial species that live within the gut of their host (Grond *et al.*, 2018). Different environments, including human-altered ones, contain different microbial communities that can colonize the gut of the host. As a result, the gut microbiota of the host can change in response to changes in land use, including urbanization (Phillips *et al.*, 2018; Teyssier *et al.*, 2018; Knutie *et al.*, 2019; Berlow *et al.*, 2021), and pollution of water and soil (Jin *et al.*, 2017). In turn, the gut microbiota can interact with host cells to change host physiological traits, such as the immune system (reviewed in Round and Mazmanian, 2009);

63 increased bacterial diversity and the presence of specific bacterial taxa can be related to increased
64 resistance to virulent parasites and pathogens. As human activity increases, along with the
65 concern over parasite-causing diseases in birds, disentangling these relationships is important to
66 understand how novel stressors can influence birds and help aid in their management and
67 conservation.

68 The Galápagos Islands of Ecuador are relatively pristine but are facing increasing human
69 activity, which allows for the study of host-associated microbiota and immune systems in
70 response to urbanization. Over the past 20 years, ecotourism and the permanent resident human
71 population has grown rapidly on the Islands (Walsh and Mena 2016). For example, from 2007 to
72 2017, the number of permanent human residents increased from approximately 20,000 to 30,000
73 people and tourist numbers increased from 140,000 to 225,000 people (Watkins and Cruz, 2007;
74 Walsh and Mena, 2016). Consequently, in areas with a human presence (e.g., towns and popular
75 tourist sites), the natural habitat and diet of endemic species have been altered with the
76 introduction of agricultural plants and human processed food (de León *et al.*, 2018). Studies have
77 shown that changes in habitat type can affect gut microbiota and metrics of immunity of
78 Darwin's finches (Zylberberg *et al.*, 2013; Michel *et al.*, 2018; Knutie *et al.*, 2019; Loo *et al.*,
79 2019). However, all studies on the gut microbiota of Darwin's finches have occurred since 2016
80 and so we know little about how earlier years of human activity has influenced microbiota in
81 Darwin's finches. Furthermore, the effect of human-influenced land use on the interaction
82 between gut microbiota and immunity has received little attention. A better understanding of
83 human impacts on avian immune systems could be especially important because introduced
84 parasites, such as avian pox and *Philornis downsi*, have been wreaking havoc on the survival of
85 birds in the islands (Wikelski *et al.*, 2004; Fessl *et al.*, 2010; Koop *et al.*, 2016).

86 The goal of this study was to determine the effect of land use on the immune response,
87 gut microbiota, and body measurements of Darwin's finches in 2008. Specifically, we
88 characterized the gut microbiota (bacterial diversity, community structure and membership, and
89 relative abundance of bacterial taxa), immune response (lysozyme activity, and haptoglobin,
90 complement antibody, and natural antibody levels), and body measurements (body mass, tarsus
91 length, and scaled mass index) in small ground finches (*Geospiza fuliginosa*) and medium ground
92 finches (*G. fortis*) across three different habitat types in 2008 on Santa Cruz Island in the
93 Galápagos. Specifically, we caught finches in three different areas that varied in human activity
94 and presence including an area with: 1) little human activity and no permanent human population
95 (hereon, undeveloped), 2) developed land for agricultural activities but only a small permanent
96 human population (hereon, agricultural), and 3) developed land for a substantial, permanent
97 human population (hereon, urban).
98 Previous studies have shown that the immune response of Darwin's finches can vary
99 temporally across land use types. For example, Zylberberg *et al.* (2013) found that finches living
100 in agricultural and undeveloped areas had different immune responses across years, whereas
101 finches in urban areas showed no change across years. One explanation is that food availability
102 changed across years but not in all habitats. Variation in food availability could either directly
103 affect the immune response or the gut microbiota, which in turn would affect the immune
104 response. However, Zylberberg et al. (2013) did not examine the effect of land use on the
105 immune response within each year. Thus, for the present study, we examined the impact of land
106 use on four metrics of immunity: lysozyme activity, and complement antibody, natural antibody,
107 and haptoglobin (using PIT54 acute phase proteins) levels. We chose these four aspects of the
108 innate immune system because each plays an integral and complementary role in the first line of

109 defense against novel pathogens (as previously discussed in Zylberberg *et al.*, 2013). In short, the
110 complement system of proteins activates the lysis of foreign cells, enhances antibody activity,
111 and directly destroys viruses (Hirsch, 1982; Murphy *et al.*, 2017). Natural antibodies bind novel
112 pathogens, facilitate phagocytosis, and promote cell lysis (Casali and Schettino, 1996; Caroll and
113 Prodeus, 1998). The PIT54 protein minimizes self-damage during inflammation and stimulates
114 the white blood cell response upon pathogen exposure (Wicher and Fries, 2006; Quaye, 2008).
115 Lysozymes lyse gram positive bacteria (Millet *et al.*, 2007). Together, these measures afford a
116 broad view of the innate immune system by providing information on both inducible and
117 constitutive components of innate immunity.

118 We hypothesized that variation in land use would influence the immune response and gut
119 microbiota of finches. Specifically, we predicted that finches in areas with lower human activity
120 (agricultural and undeveloped areas) will have more similar immune responses and gut
121 microbiota than finches living in areas with higher human activity (urban areas). Furthermore,
122 because studies have shown that the metrics of the immune system can relate to changes in the
123 gut microbiota community (Round and Mazmanian, 2009), we hypothesize that bacterial
124 diversity will relate to immune measures in the finches. Finally, because food availability and
125 thus diet differs across sites, we predict that body size (mass and tarsus length) and condition
126 (scaled mass index; Peig and Green, 2009), will differ across land use type, as found in Knutie *et*
127 *al.* (2019). Overall, our study will provide insight into the effect of human activity on the gut
128 microbiota and immune proxies of island birds, which could have conservation implications for
129 Darwin's finches (Ohmer et al., 2021).

130

131 **METHODS**

132 **Field site**

133 We conducted this study on Santa Cruz Island, Galápagos, Ecuador during the breeding
134 season in February 2008. To examine the effect of land use patterns on microbiota, immune
135 response, and body size of finches, we sampled small ground finches and medium ground finches
136 at six sites, spanning three different land use types (urban, agricultural, and undeveloped). We
137 selected the study sites to represent the range of vegetation types and precipitation present in each
138 land use and elevation category. The urban site was in Puerto Ayora (population < 20,000 in
139 2007; (Watkins and Cruz, 2007), in the lowland arid zone. Three sites were located within the
140 agricultural zone of the island, which contains a combination of small farms, fruit plantations and
141 cattle ranches. One agricultural site was in the lowlands and two in the highlands. Two sites were
142 in undeveloped areas, representing both arid lowland and moist highland zones. The Galápagos is
143 well known for extreme fluctuations in precipitation as a result of the El Niño weather pattern;
144 however, 2008 was neither particularly wet nor particularly dry on average (Zylberberg *et al.*,
145 2013).

146

147 **Sample collection**

148 To obtain immune function and microbiota data, we caught birds in mist nets that were
149 actively watched. Birds were removed from the mist net and processed as quickly as possible; in
150 the event that multiple birds entered the mist net, they were removed and kept in cloth bags until
151 they were banded and a blood sample collected. Body mass (g) and tarsus length (mm) were
152 measured, and a scaled mass index (a metric of body condition) was calculated based on the
153 methods from Peig and Green (2009). We banded each bird and collected blood samples using
154 heparinized microcapillary tubes. For birds that defecated during handling, the fecal sample was

155 immediately collected and placed in formalin until used for the fecal bacterial DNA extraction at
156 the University of Connecticut in 2019. Blood samples were kept on ice (for 4-6 hours) until they
157 were centrifuged to separate the red blood cells from the plasma. Plasma was then frozen at -
158 20°C.
159
160 **Immune measures**
161 We used a hemolysis-hemagglutination assay to measure levels of natural antibodies
162 (lysis activity) and complement antibodies (agglutination activity) (Matson *et al.*, 2005). We used
163 a commercial kit (from Tri-delta Diagnostics Inc., Morris plains, NJ, USA) to determine the
164 plasma concentration of PIT54 acute phase protein (haptoglobin levels) following Millet *et al.*
165 (2007). We used the lyso-plate assay described in Millet *et al.* (2007) to measure levels of
166 lysozyme activity in plasma samples. Each of these assays was carried out with previously
167 described protocols (Zylberberg *et al.*, 2014).
168
169 **Bacterial DNA extraction and sequencing**
170 Before starting the extraction from the finch feces, samples were centrifuged for 10
171 minutes at 10,000 rpm at 4°C and the supernatant (i.e., the formaldehyde) was then removed.
172 Nanopure water (500 uL) was added, and the sample was vortexed and centrifuged again for 5
173 minutes at 4°C at 10,000 rpm. The supernatant was removed and the step was repeated. Nanopure
174 water (200 uL) was then added to the sample and the sample was vortexed. Total DNA was
175 extracted from finch feces using a Qiagen PowerFecal DNA Isolation Kit. The protocol listed in
176 the kit was followed with the exception of the heating step; samples were heated for 30 minutes
177 at 65°C. DNA extracts were then sent to the University of Connecticut Microbial Analysis,

178 Resources and Services for sequencing with an Illumina MiSeq platform and v2 2x250 base pair
179 kit (Illumina, Inc.). We also amplified a laboratory blank to control for kit contamination and had
180 no detectable product. Bacterial inventories were conducted by amplifying the V4 region of the
181 16S rRNA gene using primers 515F and 806R (Caporaso *et al.*, 2012) and with Illumina adapters
182 and dual indices (Kozich *et al.*, 2013). Raw sequences were demultiplexed with onboard
183 bcl2fastq and then processed in Mothur v.1.42.3 (Schloss *et al.*, 2009) according to the standard
184 MiSeq protocol (Kozich *et al.*, 2013). Briefly, forward and reverse sequences were merged. All
185 sequences with any ambiguities, that did not align to the correct region, or that did not meet
186 length expectations, were removed. Sequences were aligned to the Silva nr_v128 alignment
187 (Quast *et al.*, 2013). Chimeric reads were also removed using UCHIME (Edgar *et al.*, 2011).
188 Non-bacterial sequences that are classified as chloroplasts, mitochondria, or unknown (i.e., did
189 not classify to the level of kingdom) were removed. Sequences were grouped into operational
190 taxonomic units (OTUs) based on a 97% similarity level and identification of the OTUs was done
191 using the Ribosomal Database Project Bayesian classifier (Wang *et al.*, 2007) against the Silva
192 nr_v128 taxonomy database. Alpha and beta diversity statistics were calculated by averaging
193 1,000 random subsampling of 4,000 reads per sample. We calculated observed species richness
194 (sobs) and evenness, Shannon diversity index, and Simpson diversity index. Sobs describes the
195 number of observed species and evenness describes the distribution of abundance across the
196 species. The Shannon diversity index and Simpson index are estimators of species richness and
197 species evenness but species richness is weighted more for Shannon and species evenness is
198 weighted more for Simpson. The resulting data sets included a total of 1,827,365 sequences and
199 an average of 25,032 reads per sample (min: 4,079, max: 121,506).
200

201 **Statistical analyses**

202 Generalized linear models (GLMs) were used to determine the effect of land use on
203 bacterial diversity metrics (sobs, Shannon Index, Simpson Index, and evenness), immune metrics
204 (lysozyme activity, and haptoglobin, complement antibody, and natural antibody levels), and
205 body size and condition (body mass, tarsus length, and scaled mass index). Shapiro-Wilks tests
206 were used to determine whether the variables met normality; if the variables did not meet
207 normality, they were log transformed for the analyses. Analyses were conducted using the glm
208 function within the lme4 package (Bates *et al.*, 2015). Probability values were calculated using
209 log-likelihood ratio tests using the Anova function in the car package (Fox and Weisberg, 2019).
210 Analyses were conducted in R (2021, version 4.0.4) and RStudio (2021, version 1.4.1103).

211 The effects of location and species on bacterial community dynamics were examined
212 using the Bray-Curtis dissimilarity and Jaccard similarity matrices. The matrices were created
213 using the 'vegdist' function in the vegan package in R (Oksanen *et al.*, 2019). The 'adonis2'
214 function in the vegan package was used to perform PERMANOVA to assess the differences in
215 bacterial community composition and membership between different species and locations
216 (Oksanen *et al.*, 2019). Both Bray-Curtis and Jaccard are dissimilarity matrices, with Bray-Curtis
217 taking into account the relative abundances of shared taxa (community structure) while Jaccard
218 only considers the presence or absence of such taxa (community membership). Principal
219 Coordinates Analyses (PCoA), where the distances among the samples are converted onto a
220 graph, were done to compare and visualize differences between groups.

221 Relative abundances (arcsine square root transformed; (Shchipkova *et al.*, 2010; Kumar *et*
222 *al.*, 2012) of bacterial phyla and genera were compared between species and location groups.
223 Data were manipulated using packages tidyr, reshape2, and plyr in R (Wickham, 2007, 2011;

224 Wickham and Henry, 2019) and ANOVAs were run in the car package in R (Fox and Weisberg,
225 2019); false discovery rate (FDR) tests were used to control for multiple analyses. A Tukey post-
226 hoc test was done to determine which land use types differed from each other. All figures were
227 created in Prism (2020, version 9).
228
229 **RESULTS**
230 **Effect of land use and finch species on the gut microbiota**
231 Bacterial diversity, as measured by sobs, differed among land use types for finches (Table
232 S1; Fig. 1A). Birds in the urban area had higher sobs values than birds in agricultural and
233 undeveloped areas (Table S2; $P < 0.05$). However, there was no effect of species or an interaction
234 between species and land use type on sobs values (Table S1). Land use, bird species, and their
235 interaction did not affect Shannon Index, Shannon evenness, and the Simpson Index (Tables S1-
236 S2).
237 Bacterial community structure ($F = 1.33$, $P = 0.04$) and membership ($F = 1.19$, $P = 0.045$)
238 differed between urban areas compared to the other land use types for medium ground finches
239 (Fig. 2A-B). However, bacterial community structure ($F = 0.71$, $P = 0.98$) and membership ($F =$
240 0.83, $P = 0.98$) did not differ significantly among land uses in small ground finches (Fig. 2A-B).
241 For small ground finches, relative abundances of several genera, including genera
242 *Pseudoxanthomonas*, *Cloacibacterium*, and *Dietzia*, were higher in urban areas compared to the
243 other two areas (Table S3). Relative abundances of phyla did not differ among land use types for
244 small or medium ground finches ($P > 0.05$). Relative abundances of genera did not differ among
245 land use types for medium ground finches either ($P > 0.05$).
246

247 **Effect of land use and finch species on the immune response**

248 Lysozyme activity differed among land use types for the medium and small ground
249 finches (Fig. 1B; Table S4). Overall, urban finches had statistically lower lysozyme levels than
250 finches in the agricultural and undeveloped areas, but this effect was more pronounced in
251 medium ground finches (Fig. 1B; Table S5). Haptoglobin, complement antibody, and natural
252 antibody levels did not differ significantly among land use types in either medium or small
253 ground finches (Table S4-S5). Immune metrics did not correlate significantly with the bacterial
254 diversity metrics ($R^2 < 0.20$ for all pairs).

255

256 **Effect of land use and finch species on body size and mass**

257 Body mass differed between small and medium ground finches (Fig. 3; Table S6-S7). For
258 small ground finches, body mass did not differ significantly among land use types, but for
259 medium ground finches, individuals in the undeveloped area had lower body mass than
260 individuals in agricultural and urban areas (Fig. 3; Table S7). Tarsus length and scaled mass
261 index differed significantly between species but not among land use types (Table S6-S7).

262

263 **DISCUSSION**

264 Our study examined the effect of land use on the gut microbiota, immune metrics, and
265 body measurements of two Darwin's finch species in 2008, at the start of a rapid increase in
266 human activity in the Galápagos Islands. Studies of samples collected in 2016 have found
267 significant impacts of human activity on metrics of the gut microbiota and body measurements of
268 Darwin's finches. Based on data collected eight years earlier, we found marginal effects of
269 urbanization on the observed species richness (sobs) across bird species and a few bacterial

genera (*Pseudoxanthomonas*, *Cloacibacterium*, and *Dietzia*) in small ground finches. Similarly, we found few effects of land use on immune metrics and body measurements, with urbanization affecting lysozyme levels across bird species and body mass in medium ground finches. Furthermore, bacterial diversity did not correlate with any of the immune measures. Although humans have had a permanent presence in the Galápagos for decades, our results suggest that initially, increased human activity (starting ~2007) had marginal effects on the finches.

Lysozyme activity, but not the other immune metrics, was influenced by land use type; urban birds had lower lysozyme activity than non-urban birds. Lysozymes are part of the innate immune system, which are transferred from mother to egg whites. During the breeding season, lysozyme activity declines in females during pre-laying and egg laying (Saino *et al.* 2002). Urban Darwin's finches differ in the length and timing of their breeding season compared to non-urban birds (Harvey *et al.* 2021). Samples were collected from female finches in February, which is early in the breeding season. Therefore, timing of breeding could be responsible for differences in lysozyme activity in urban vs. non-urban birds. Furthermore, although lysozyme is an antimicrobial enzyme, we found no relationship between lysozyme activity and bacterial diversity. This lack of relationship is likely because the lysozymes quantified in our study were in the plasma, rather in the gut mucosal tissue. Overall, because lysozyme activity differs across land uses in 2008, early in urbanization, future studies could focus on how lysozyme activity has changed in finches since then, especially in response to emerging diseases (Wikelski *et al.* 2004).

Observed species richness (sobs) was higher in urban areas compared to non-urban areas. However, the other alpha diversity metrics that consider species evenness did not differ across habitats. Other studies that have found higher observed species richness and Shannon index in urban birds indicate that this could be caused by man-made landscapes and a higher grass and

293 tree cover (Phillips *et al.*, 2018; Berlow *et al.*, 2021). However, there have also been studies
294 demonstrating less bacterial diversity in species living in an urban habitat when compared to
295 species living in a non-urban habitat (Barelli *et al.*, 2015; Teyssier *et al.*, 2018, 2020; Knutie *et*
296 *al.*, 2019). A less diverse gut community in urban areas could be caused by habitat disturbances
297 or differences in diets between urban and non-urban populations (Sonnenburg *et al.*, 2016;
298 Teyssier *et al.*, 2018, 2020; Knutie, 2020). The time of sampling should also be considered, as
299 Tessyier *et al.* (2018) found that birds in a non-urban habitat exhibited less microbiome diversity
300 during the winter months, while microbiome diversity in birds in an urban habitat did not differ
301 across seasons. As the birds included in this study were sampled at the start of the rainy/breeding
302 season, it is possible that a seasonal effect contributed to the difference seen between birds from
303 non-urban and urban habitats. Overall, our results contribute to the growing body of literature
304 demonstrating that there are many factors to consider when studying the relationship between
305 urbanization and gut microbiome diversity.

306 Relative abundances of several genera, including *Pseudoxanthomonas* (phylum
307 Proteobacteria), *Cloacibacterium* (phylum Bacteroidetes), and *Dietzia* (phylum Actinobacteria),
308 were higher in urban areas than non-urban areas in small ground finches in 2008. Studies have
309 not specifically documented differences in these genera across land use types but have found
310 higher relative abundances of family Weeksellaceae (includes *Cloacibacterium spp.*) in urban
311 areas compared to non-urban areas (Berlow *et al.*, 2021). Interestingly, small ground finches in
312 2016 only showed increases in the relative abundance of *Steroidobacter spp.* in the presence of
313 humans (Knutie *et al.*, 2019). Although we found no effect of human activity on bacterial phyla
314 in 2008 finches, 2016 finches in areas with higher human activity had higher relative abundances
315 of phyla Chlamydiae and Cyanobacteria than in other areas.

316 One explanation for differences in bacterial taxa between 2008 and 2016 finches is that
317 the gut microbiota of finches has changed over eight years coinciding with a change in the
318 finches' environment. Davidson et al., (2020) found that relative abundances of phyla
319 Proteobacteria, Bacteroidetes, Actinobacteria in great tits (*Parus major*) were higher in urban
320 areas compared to rural areas and/or when tits were fed an insect-based diet compared to a seed-
321 based diet. Recent studies have found that urban small and medium ground finches tend to prefer
322 human food diets (e.g., chips and cooked rice) rather than their natural diet (e.g., seeds) which
323 could explain our results (de León *et al.*, 2018). Alternatively, urban contaminants could be
324 responsible for changes in the gut bacterial taxa observed in the finches. *Pseudoxanthomonas*
325 *spp.* have been found in oil-polluted water bodies and soil because the bacterial taxa can
326 metabolize and degrade the contaminant (Young *et al.*, 2007). Although human activity in the
327 Islands did not rapidly increase until approximately 2007, sources of human-driven pollution
328 were present in towns such as Puerto Ayora. For example, the major presence of vehicles began
329 in the 2000s with the completion of major paved roads. Although contaminants can affect the
330 abundance of bacterial taxa, medium ground finches were likely exposed to the same
331 environmental contaminants as the small ground finches but, nonetheless, did not show
332 differences in bacterial taxa across land uses. However, since small and medium ground finches
333 do eat different natural diets, perhaps the natural diet of the small ground finches shifted in
334 response to urbanization over time, while the medium ground finches did not.
335 Body mass differed across land-use types for medium ground finches, with non-urban
336 birds having lower body mass than those in urban areas. These results were also found by
337 McNew *et al.* (2017) and can potentially be explained by differences in food availability across
338 sites; human activity can affect food availability and preference for wildlife. For example, the

339 diet of non-urban birds includes mostly natural foods, such as seeds, fruits, and insects, while
340 urban birds can prefer human processed food (Murray *et al.*, 2016; de León *et al.*, 2018; Phillips
341 *et al.*, 2018). A human-based diet can affect body morphometrics of animals, including increased
342 body mass (Banks and Dickman, 2000; Bayol *et al.*, 2007; Wilcoxen *et al.*, 2015). In contrast, we
343 found no effect of land use on the body mass of small ground finches. Small ground finches have
344 smaller bill sizes (length, width, depth) and therefore different diets than medium ground finches
345 (Abbott *et al.*, 1977). One explanation is that perhaps medium ground finches are better able to
346 exploit the urban diet than small ground finches. Interestingly, small ground finches in areas with
347 more human activity in 2016 were larger than in areas with no human activity, which might be
348 related to the increase in human activity over time. Since museum specimens and long-term field
349 data exist, a future study could determine the effect of human activity on different finch species
350 in urban and non-urban areas across islands.
351
352 **CONCLUSION**
353 Our study suggests that immunity, gut microbiota, and body mass of two species of
354 Darwin's finches vary across land use prior to the rapid increase in human activity in Galápagos
355 Islands. These results suggest that earlier human activity can affect the ecology of birds. Over the
356 past several decades, Darwin's finches have faced increasing challenges from invasive parasites
357 (Wikelski *et al.*, 2004; B. Fessl *et al.*, 2010; Koop *et al.*, 2016; Knutie, 2018) and predators
358 (Gotanda, 2021), anthropogenic debris (Theodosopoulos and Gotanda, 2018; Harvey *et al.*,
359 2021), and dynamic annual changes in natural and novel food availability (Grant and Grant,
360 1995; de León *et al.*, 2018), which can all affect the physiology and gut microbiota of animals.
361 Given our results and the novel challenges facing the Galápagos Islands, the iconic Darwin's

362 finch system has exciting potential for future ecoimmunology and microbiome research in a
363 changing world (Ohmer *et al.*, 2021).
364

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375 applicable international, national, and/or institutional guidelines for the care and use of animals
376 were followed (UC Davis IACUC protocol 13171).
377

378 **Authors' contributions**

379 MZ and SAK conceived the study, MZ collected the samples and field data, MZ and AA
380 conducted the laboratory work, AA conducted the bioinformatics, JNB, AA, and SAK did the
381 data analyses. All authors wrote, revised, and approved the manuscript.
382

383 **Data accessibility**

Data are available at FigShare (doi: available upon acceptance) and sequences have been uploaded to GenBank (BioProject accession number: available upon acceptance).

Conflict of Interest

The authors declare that they have no conflict of interest.

LITERATURE CITED

Abbott I, Abbott LK, Grant PR (1977) Comparative Ecology of Galápagos Ground Finches (Geospiza Gould): Evaluation of the Importance of Floristic Diversity and Interspecific Competition. *Ecological Monographs* 47. doi:10.2307/1942615

Atwell JW, Cardoso GC, Whittaker DJ, Campbell-Nelson S, Robertson KW, Ketterson ED (2012) Boldness behavior and stress physiology in a novel urban environment suggest rapid correlated evolutionary adaptation. *Behavioral Ecology* 23: 960–969.

Banks PB, Dickman CR (2000) Effects of winter food supplementation on reproduction, body mass, and numbers of small mammals in montane Australia. *Canadian Journal of Zoology* 78. doi:10.1139/z00-110

Barelli C, Albanese D, Donati C, Pindo M, Dallago C, Rovero F, Cavalieri D, Michael Tuohy K, Christine Hauffe H, de Filippo C (2015) Habitat fragmentation is associated to gut microbiota diversity of an endangered primate: Implications for conservation. *Scientific Reports* 5. doi:10.1038/srep14862

Bates D, Mächler M, Bolker BM, Walker SC (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*. doi:10.18637/jss.v067.i01

406 Bayol SA, Farrington SJ, Stickland NC (2007) A maternal "junk food" diet in pregnancy and
407 lactation promotes an exacerbated taste for "junk food" and a greater propensity for obesity
408 in rat offspring. *British Journal of Nutrition* 98: 843–51.
409 Berlow M, Phillips JN, Derryberry EP (2021) Effects of Urbanization and Landscape on Gut
410 Microbiomes in White-Crowned Sparrows. *Microbial Ecology* 81. doi:10.1007/s00248-
411 020-01569-8
412 Bosse M, Spurgin LG, Laine VN, Cole EF, Firth JA, Gienapp P, Gosler AG, McMahon K,
413 Poissant J, Verhagen I, *et al.* (2017) Recent natural selection causes adaptive evolution of
414 an avian polygenic trait. *Science* 6361: 365–368.
415 Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N (2012) Ultra-high-
416 throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms.
417 *ISME J* 6. doi:10.1038/ismej.2012.8
418 Caroll MC, Prodeus AP (1998) Linkages of innate and adaptive immunity. *Current Opinion in*
419 *Immunology* 10. doi:10.1016/S0952-7915(98)80028-9
420 Casali P, Schettino EW (1996) Structure and function of natural antibodies. *Current Topics in*
421 *Microbiology and Immunology* 210. doi:10.1007/978-3-642-85226-8_17
422 Cornet S, Bichet C, Larcombe S, Faivre B, Sorci G (2014) Impact of host nutritional status on
423 infection dynamics and parasite virulence in a bird-malaria system. *The Journal of Animal*
424 *Ecology* 83: 256–265.
425 Davidson GL, Wiley N, Cooke AC, Johnson CN, Fouhy F, Reichert MS, de la Hera I, Crane
426 JMS, Kulahci IG, Ross RP, *et al.* (2020) Diet induces parallel changes to the gut microbiota
427 and problem solving performance in a wild bird. *Scientific Reports* 10. doi:10.1038/s41598-
428 020-77256-y

429 de León L, Sharpe D, Gotanda K, Raeymaekers J, Chaves J, Hendry A, Podos J (2018)
430 Urbanization erodes niche segregation in Darwin's finches. *Evolutionary Applications*.
431 Demas GE (2004) The energetics of immunity: a neuroendocrine link between energy balance
432 and immune function. *Hormones and Behavior*.
433 Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and
434 speed of chimera detection. *Bioinformatics* 27: 2194–2200.
435 Fessl Birgit, Young GH, Young RP, Rodríguez-Matamoros J, Dvorak M, Tebbich S, Fa JE
436 (2010) How to save the rarest Darwin's finch from extinction: the mangrove finch on
437 Isabela Island. *Philosophical Transactions of the Royal Society of London B* 365: 1019–
438 1030.
439 Fessl B., Young GH, Young RP, Rodríguez-Matamoros J, Dvorak M, Tebbich S, Fa JE (2010)
440 How to save the rarest Darwin's finch from extinction: the mangrove finch on Isabela
441 Island. *Philosophical Transactions of the Royal Society of London B* 365: 1019–1030.
442 Fox J, Weisberg S (2019) CAR - An R Companion to Applied Regression. Thousand Oaks CA:
443 Sage.
444 Gotanda KM (n.d.) Human influences on antipredator behaviour in Darwin's finches.
445 Grant PR, Grant BR (1995) Predicting microevolutionary responses to directional selection on
446 heritable variation. *Evolution* 49: 241–251.
447 Grond K, Sandercock BK, Jumpponen A, Zeglin LH (2018) The avian gut microbiota:
448 community, physiology and function in wild birds. *Journal of Avian Biology* 49: e01788.
449 Harvey JA, Chernicky K, Simons SR, Verrett TB, Chaves JA, Knutie SA (2021) Urban living
450 influences the nesting success of Darwin's finches in the Galápagos Islands. *Ecology and*
451 *Evolution* 11. doi:10.1002/ece3.7360

452 Hendry AP, Grant PR, Rosemary Grant B, Ford HA, Brewer MJ, Podos J (2006) Possible human
453 impacts on adaptive radiation: beak size bimodality in Darwin's finches. *Proceedings of the*
454 *Royal Society B: Biological Sciences* 273: 1887–94.
455 Hirsch RL (1982) The complement system: Its importance in the host response to viral infection.
456 *Microbiological Reviews*.
457 Howick VM, Lazzaro BP (2014) Genotype and diet shape resistance and tolerance across distinct
458 phases of bacterial infection. *BMC Evolutionary Biology* 14: 56.
459 Jin Y, Wu S, Zeng Z, Fu Z (2017) Effects of environmental pollutants on gut microbiota.
460 *Environmental Pollution*.
461 Knutie SA (2018) Relationships among introduced parasites, host defenses, and gut microbiota of
462 Galápagos birds. *Ecosphere* 9: e02286.
463 Knutie SA (2020) Food supplementation affects gut microbiota and immunological resistance to
464 parasites in a wild bird species. *Journal of Applied Ecology* 57. doi:10.1111/1365-
465 2664.13567
466 Knutie SA, Chaves JA, Gotanda KM (2019) Human activity can influence the gut microbiota of
467 Darwin's finches in the Galápagos Islands. *Molecular Ecology* mec.15088.
468 Koop JAH, Kim PS, Knutie SA, Adler F, Clayton DH (2016) An introduced parasitic fly may
469 lead to local extinction of Darwin's finch populations. *Journal of Applied Ecology* 53.
470 doi:10.1111/1365-2664.12575
471 Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD (2013) Development of a dual-
472 index sequencing strategy and curation pipeline for analyzing amplicon sequence data on
473 the miseq illumina sequencing platform. *Applied and Environmental Microbiology* 79:
474 5112–5120.

475 Kumar PS, Mason MR, Brooker MR, O'Brien K (2012) Pyrosequencing reveals unique microbial
476 signatures associated with healthy and failing dental implants. *Journal of Clinical*
477 *Periodontology* 39: 425–433.
478 Lochmiller RL, Deerenberg C (2000) Trade-Offs in Evolutionary Immunology: just what Is the
479 cost of immunity? *Oikos* 88: 87–98.
480 Loo WT, García-Loor J, Dudaniec RY, Kleindorfer S, Cavanaugh CM (2019) Host phylogeny,
481 diet, and habitat differentiate the gut microbiomes of Darwin's finches on Santa Cruz
482 Island. *Scientific Reports* 9. doi:10.1038/s41598-019-54869-6
483 Matson KD, Ricklefs RE, Klasing KC (2005) A hemolysis-hemagglutination assay for
484 characterizing constitutive innate humoral immunity in wild and domestic birds.
485 *Developmental and Comparative Immunology* 29. doi:10.1016/j.dci.2004.07.006
486 McNew SM, Beck D, Sadler-Riggleman I, Knutie SA, Koop JAH, Clayton DH, Skinner MK
487 (2017) Epigenetic variation between urban and rural populations of Darwin's finches. *BMC*
488 *Evolutionary Biology* 17. doi:10.1186/s12862-017-1025-9
489 Michel AJ, Ward LM, Goffredi SK, Dawson KS, Baldassarre DT, Brenner A, Gotanda KM,
490 McCormack JE, Mullin SW, O'Neill A, *et al.* (2018) The gut of the finch: uniqueness of the
491 gut microbiome of the Galápagos vampire finch. *Microbiome* 6: 167.
492 Millet S, Bennett J, Lee KA, Hau M, Klasing KC (2007) Quantifying and comparing constitutive
493 immunity across avian species. *Developmental & Comparative Immunology* 31: 188–201.
494 Møller AP (2008) Flight distance of urban birds, predation, and selection for urban life.
495 *Behavioral Ecology and Sociobiology* 63: 63.
496 Murphy K, Weaver C, Janeway C (2017) Janeway 's Immunobiology 9Th Edition. America.

497 Murray MH, Becker DJ, Hall RJ, Hernandez SM (2016) Wildlife health and supplemental
498 feeding: A review and management recommendations. *Biological Conservation* 204: 163–
499 174.
500 Ohmer MEB, Costantini D, Czirják G, Downs CJ, Ferguson L v., Flies A, Franklin CE, Kayigwe
501 AN, Knutie S, Richards-Zawacki CL, *et al.* (2021) Applied ecoimmunology: Using
502 immunological tools to improve conservation efforts in a changing world. *Conservation*
503 *Physiology*.
504 Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, Mcglinn D, Minchin PR, O'Hara RB,
505 Simpson GL, Solymos P, *et al.* (2019) vegan: Community Ecology Package. R package
506 version 2.4-2. *Community ecology package* 2.5-6.
507 Peig J, Green AJ (2009) New perspectives for estimating body condition from mass/length data:
508 the scaled mass index as an alternative method. *Oikos* 118: 1883–1891.
509 Phillips JN, Berlow M, Derryberry EP (2018) The effects of landscape urbanization on the gut
510 microbiome: an exploration into the gut of urban and rural white-crowned sparrows.
511 *Frontiers in Ecology and Evolution* 6: 1–10.
512 Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The
513 SILVA ribosomal RNA gene database project: improved data processing and web-based
514 tools. *Nucleic Acids Research* 41: D590–D596.
515 Quaye IK (2008) Haptoglobin, inflammation and disease. *Transactions of the Royal Society of*
516 *Tropical Medicine and Hygiene*.
517 Round June L, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses
518 during health and disease. *Nature Reviews Immunology* 9: 313–323.

519 Round June L., Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses
520 during health and disease. *Nature Reviews Immunology* 9: 313–323.
521 Samia DSM, Blumstein DT, Díaz M, Grim T, Ibáñez-Álamo JD, Jokimäki J, Tätte K, Markó G,
522 Tryjanowski P, Møller AP (2017) Rural-Urban Differences in Escape Behavior of European
523 Birds across a Latitudinal Gradient. *Frontiers in Ecology and Evolution* 5: 1–13.
524 Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley
525 BB, Parks DH, Robinson CJ, *et al.* (2009) Introducing mothur: Open-source, platform-
526 independent, community-supported software for describing and comparing microbial
527 communities. *Applied and Environmental Microbiology* 75: 7537–7541.
528 Shchipkova AY, Nagaraja HN, Kumar PS (2010) Subgingival microbial profiles of smokers with
529 periodontitis. *Journal of Dental Research* 89: 1247–1253.
530 Sheldon BC, Verhulst S (1996) Ecological immunology: costly parasite defences and trade-offs in
531 evolutionary ecology. *Trends in Ecology & Evolution* 11: 317–321.
532 Sol D, Maspons J, Gonzalez-Voyer A, Morales-Castilla I, Garamszegi LZ, Møller AP (2018)
533 Risk-taking behavior, urbanization and the pace of life in birds. *Behavioral Ecology and*
534 *Sociobiology* 72: 58–66.
535 Sonnenburg ED, Smits SA, Tikhonov M, Higginbottom SK, Wingreen NS, Sonnenburg JL
536 (2016) Diet-induced extinctions in the gut microbiota compound over generations. *Nature*
537 529. doi:10.1038/nature16504
538 Start D, Bonner C, Weis AE, Gilbert B (2018) Consumer-resource interactions along urbanization
539 gradients drive natural selection. *Evolution* 9: 1863–1873.

540 Sternberg ED, Lefèvre T, Li J, de Castillejo CLF, Li H, Hunter MD, de Roode JC (2012) Food
541 plant derived disease tolerance and resistance in a natural butterfly-plant-parasite
542 interactions. *Evolution* 66: 3367–3376.
543 Strandin T, Babayan SA, Forbes KM (2018) Reviewing the effects of food provisioning on
544 wildlife immunity. *Philosophical Transactions of the Royal Society B: Biological Sciences*
545 373: 20170088.
546 Svensson E, Råberg L, Koch C, Hasselquist D (1998) Energetic stress, immunosuppression and
547 the costs of an antibody response. *Functional Ecology* 12: 912–919.
548 Teyssier A, Matthysen E, Hudin NS, de Neve L, White J, Lens L (2020) Diet contributes to
549 urban-induced alterations in gut microbiota: Experimental evidence from a wild passerine.
550 *Proceedings of the Royal Society B: Biological Sciences* 287. doi:10.1098/rspb.2019.2182
551 Teyssier A, Rouffaer LO, Saleh Hudin N, Strubbe D, Matthysen E, Lens L, White J (2018) Inside
552 the guts of the city: Urban-induced alterations of the gut microbiota in a wild passerine.
553 *Science of the Total Environment* 612: 1276–1286.
554 Theodosopoulos AN, Gotanda KM (2018) Death of a Darwin's Finch: a consequence of human-
555 made debris? *The Wilson Journal of Ornithology*. doi:10.1676/17-00050.1
556 Walsh SJ, Mena CF (2016) Interactions of social, terrestrial, and marine sub-systems in the
557 Galápagos Islands, Ecuador. *Proceedings of the National Academy of Sciences* 113: 14536–
558 14543.
559 Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naïve Bayesian classifier for rapid assignment
560 of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental*
561 *Microbiology* 73: 5261–5267.

562 Watkins G, Cruz F (2007) Galápagos at Risk: A Socioeconomic Analysis of the Situation in the
563 Archipelago. Puerto Ayora, Santa Cruz.
564 Wicher KB, Fries E (2006) Haptoglobin, a hemoglobin-binding plasma protein, is present in bony
565 fish and mammals but not in frog and chicken. *Proceedings of the National Academy of*
566 *Sciences of the United States of America* 103. doi:10.1073/pnas.0508723103
567 Wickham H (2007) Reshaping data with the reshape package. *Journal of Statistical Software* 21.
568 doi:10.18637/jss.v021.i12
569 Wickham H (2011) The split-apply-combine strategy for data analysis. *Journal of Statistical*
570 *Software* 40. doi:10.18637/jss.v040.i01
571 Wickham H, Henry L (2019) tidyr: Tidy Messy Data. *R package version 100*.
572 Wikelski M, Foufopoulos J, Vargas H, Snell H (2004) Galápagos birds and diseases: invasive
573 pathogens as threats for island species. *Ecology and Society* 9: online:
574 http://www.ecologyandsociety.org/vol9/iss1.
575 Wilcoxen TE, Horn D, Hogan BM, Hubble CN, Huber SJ, Flamm J, Knott M, Lundstrom L,
576 Salik F, Wassenhove SJ, *et al.* (2015) Effects of bird-feeding activities on the health of wild
577 birds. *Conservation Physiology* 3: cov058.
578 Young CC, Ho MJ, Arun AB, Chen WM, Lai WA, Shen FT, Rekha PD, Yassin AF (2007)
579 Pseudoxanthomonas spadix sp. nov., isolated from oil-contaminated soil. *International*
580 *Journal of Systematic and Evolutionary Microbiology* 57. doi:10.1099/ijs.0.65053-0
581 Zylberberg M, Klasing KC, Hahn TP (2014) In house finches, Haemorhous mexicanus, risk
582 takers invest more in innate immune function. *Animal Behaviour* 89.
583 doi:10.1016/j.anbehav.2013.12.021

584 Zylberberg M, Lee KA, Klasing KC, Wikelski M (2013) Variation with land use of immune
585 function and prevalence of avian pox in Galápagos finches. *Conservation Biology* 27: 103–
586 112.
587
588

589 **Figure Legends**

590

591 Fig 1. The effect of land use type (undeveloped, agricultural [Ag], urban) on mean (± SE) sobs

592 bacterial diversity (A) and lysozyme activity (B). Each point represents an individual.

593

594 Fig. 2. The effect of land use type on bacterial community structure and membership for medium

595 ground finches (A: structure, B: membership) and small ground finches (C: structure, D:

596 membership).

597

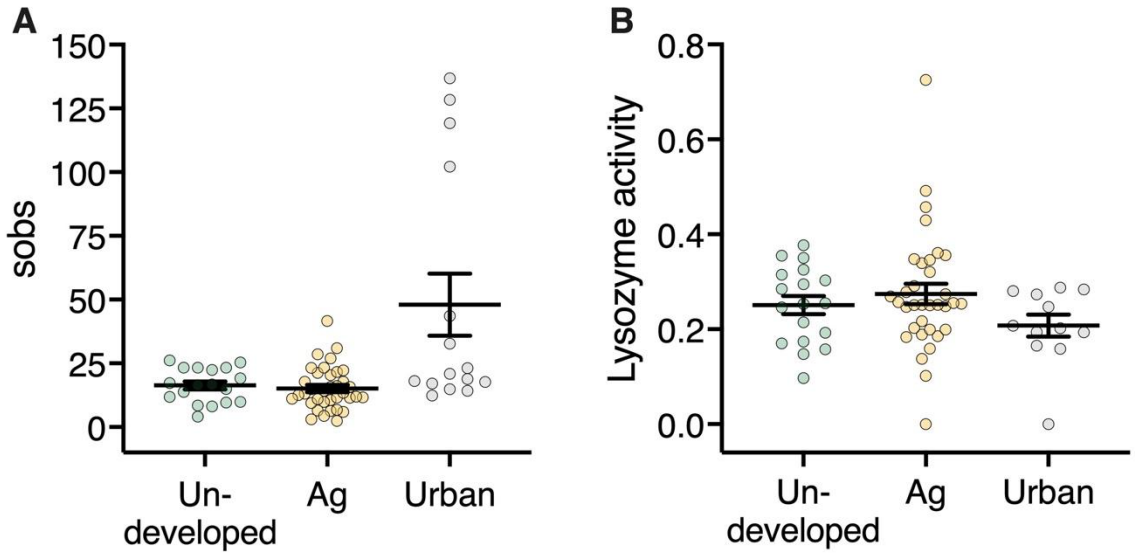
598 Fig. 3. The effect of land use type (undeveloped, agricultural [Ag], urban) and finch species

599 (small ground finch [SGF], and medium ground finch [MGF]) on body mass.

600

601

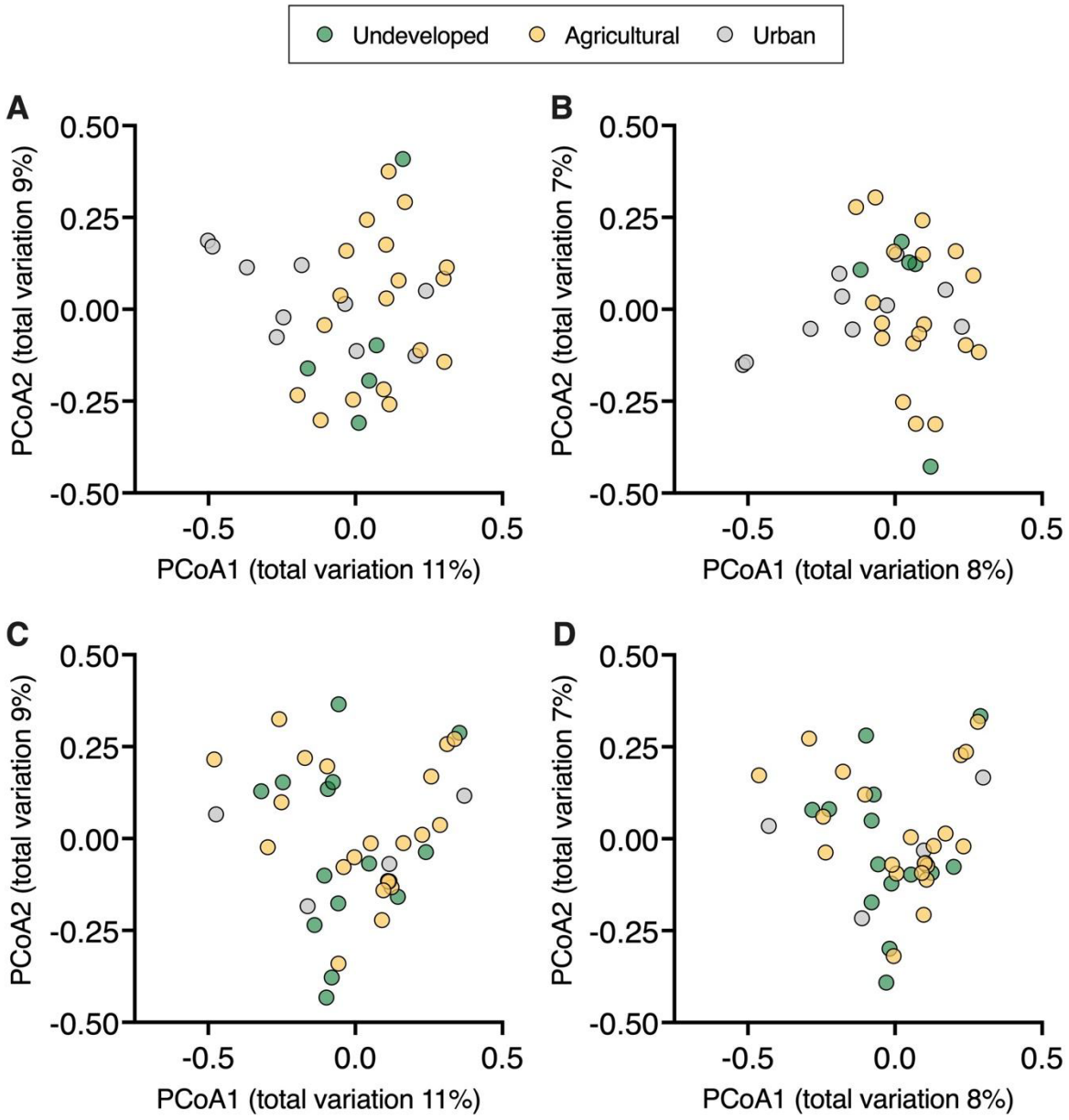
602 Fig. 1.



603

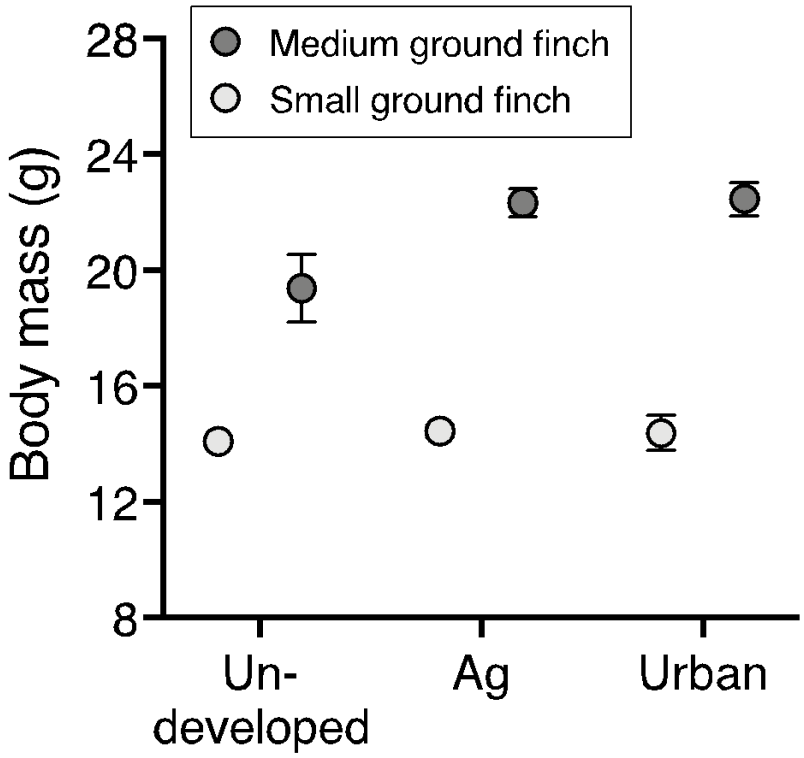
604

605 Fig. 2.


Undeveloped
Agricultural
Urban
A
B
C
D
0.50
0.25
0.00
-0.25
-0.50
-0.5
0.0
0.5
PCoA2 (total variation 9%)
PCoA1 (total variation 11%)
PCoA2 (total variation 7%)
PCoA1 (total variation 8%)

606

607 Fig. 3.


Medium ground finch
Small ground finch
Body mass (g)
28
24
20
16
12
8
Un-
developed
Ag
Urban

608