Urban living can rescue Darwin's finches from the lethal effects of invasive vampire flies

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Abstract

Human activity changes multiple factors in the environment, which can have additive or neutralizing effects on organisms. However, few studies have explored the causal effects of multiple anthropogenic factors, such as urbanization and invasive species, on animals, and the mechanisms that mediate these interactions. This study examines the influence of urbanization on the detrimental effect of invasive avian vampire flies (*Philornis downsi*) on endemic Darwin's finches in the Galápagos Islands. We experimentally manipulated nest fly abundance in an urban and non-urban area and then characterized nestling health, survival, diet, and gene expression patterns related to host defense. Survival of non-parasitized nestlings from urban (85%) and non-urban (78%) nests did not differ significantly. However, parasitized, non-urban nestlings lost more blood and had lower survival (7%) compared to urban nestlings (50%). Stable isotopic values ($\delta^{15}N$) from nestling feces revealed that diet differed between urban and non-urban nestlings. $\delta^{15}N$ values correlated negatively with parasite abundance, which suggests that diet might influence host defenses (e.g., tolerance and resistance). Parasitized urban nestlings differentially expressed genes within pathways associated with red blood cell production (tolerance) and pro-inflammatory response (innate immunological resistance), compared to parasitized non-urban nestlings. In contrast, parasitized non-urban nestlings differentially expressed genes within pathways associated with immunoglobulin production (adaptive immunological resistance). Our results suggest that urban nestlings are investing more in pro-inflammatory responses to resist parasites, but also recovering more blood cells to tolerate blood loss. Although non-urban nestlings are mounting an adaptive immune response, it is likely a last effort by the immune system rather than an effective defense against avian vampire flies since few nestlings survived.

43 **Key words:** Darwin's finch, ecoimmunology, host defenses, gene expression, invasive parasites,

44 resistance, tolerance, transcriptomics

45 **Introduction**

46 Emerging diseases are a major global threat to biodiversity (Daszak et al., 2000; Keesing
47 et al., 2010). Naïve hosts who cannot effectively defend themselves against novel disease-
48 causing parasites may risk population declines or even extinction (van Riper III & van Riper,
49 1986; Frick et al., 2010). However, not all hosts are susceptible to introduced parasites. The
50 fitness of some host species is clearly reduced, while the fitness of other hosts is relatively
51 unaffected. Less affected hosts may alleviate parasite damage with defense mechanisms, such as
52 tolerance and resistance (Read et al., 2008). Tolerance mechanisms, such as tissue repair or
53 recovery of blood loss due to parasites, minimize the damage that parasites cause without
54 reducing parasite fitness (Miller et al., 2006; Råberg et al., 2007; Read et al., 2008; Medzhitov et
55 al., 2012). For example, parents from parasite-infested nests reduce the cost of parasitism by
56 feeding their offspring more than parents from non-parasitized nests (Christe et al., 1996; Tripet
57 & Richner, 1997; Knutie et al., 2016). Consequently, despite increasing parasite loads, offspring
58 do not suffer a high cost of parasitism because they are able to compensate for resources lost to
59 the parasites.

60 Resistance, as a defense mechanism, minimizes the damage that parasites cause by
61 reducing parasite fitness (Read et al., 2008). The immune system is an example of a resistance
62 mechanism that includes the innate and adaptive immune responses. The innate immune
63 response acts as the front line of defense because it is a rapid, non-specific response to an
64 antigen. Key innate immune molecules include natural killer cells and signaling proteins (e.g.,
65 interleukins [IL] and interferons [IFN]). The adaptive immune response is a slower, more
66 specific, response to antigens but has "memory" and can more rapidly initiate during subsequent
67 antigen exposure. The key adaptive immune molecules are T helper cells (e.g., CD4+) and B
68 cells, which are involved in pathways that produce antibodies (e.g., Ig). Both innate and adaptive

69 immune responses are initiated when a host is bitten by an ectoparasite, such as a nest fly (Owen
70 et al., 2010). The innate immune response is activated first with the release of pro-inflammatory
71 cytokines (e.g., IL-6, IL-1β, and IL-17 involved in the Th1-directed pathway, IL-12, and IFNs).
72 Helper T lymphocytes (T_H) of the adaptive immune response then recognize and are activated by
73 the antigen-MHC complex on these antigen-presenting cells, which then induce the production
74 of antigen-specific antibodies by B lymphocytes. Phagocytic and granulocytic cells are then
75 recruited to the wound by T_H cells, which induce an increase in size or number of normal cells
76 and fluid at the wound site, causing inflammation. The immune cascade described above can
77 decrease ectoparasite fitness by causing edema (tissue swelling), which prevents the parasites
78 from feeding from capillaries, and can damage the parasite's tissue with proteolytic molecules
79 (Owen et al., 2010). The innate and adaptive host responses to ectoparasites can vary across
80 individual hosts and populations, which is often affected by environmental factors, such as food
81 availability (Knutie, 2020).

82 Humans in urban environments can increase food availability and reliability for animals
83 with the establishment of wild animal feeders or the impartial disposal of human trash. Due to
84 the high energetic cost of defense mechanisms, only hosts with sufficient food resources, such as
85 in these urban areas, may be able to invest in defenses (Sheldon & Verhulst, 1996; Svensson et
86 al., 1998; Lochmiller & Deerenberg, 2000; Sternberg et al., 2012; Cornet et al., 2014; Howick &
87 Lazzaro, 2014; Knutie, 2020). These extra nutrients, such as protein, can directly increase the
88 production of immune cells (Coop & Kyriazakis, 2001; Strandin et al., 2018). Consequently,
89 individuals with less effective defenses, but better access to resources, might be better equipped
90 to resist the negative effects of parasites. Alternatively, the lower nutritional quality of the
91 human-supplemented food could decrease the hosts' ability to produce an effective immune
92 response to parasites. Without the development or evolution of resistance and tolerance defenses,

hosts can face a reduction in population size or even extinction and this effect might be especially apparent in the context of human-influenced environments (van Riper III & van Riper, 1986; Atkinson & Lapointe, 2009). Understanding the effects of these complex, non-mutually exclusive interactions is critical because the movement of parasites around the world is only increasing, and the urban ecosystem is one of the few that is rapidly expanding (Birch & Wachter, 2011; Verrelli et al., 2022).

The effects of urbanization on host defenses against parasites might be particularly pronounced on islands where population sizes are small, genetic diversity is relatively low, and where species have evolved in the absence of introduced parasites (e.g., Hawaiian honeycreepers and malaria; Atkinson & Lapointe, 2009). The Galápagos Islands of Ecuador are relatively pristine but face increasing changes as a result of a growing human presence. Ecotourism and the permanent resident human population have grown exponentially in the Galápagos over several decades with nearly 225,000 visiting tourists each year and 30,000 permanent residents (Walsh & Mena, 2016). The introduction of parasites to the Galápagos is also relatively recent, such as the avian vampire fly (*Philornis downsi*), which was introduced within the past few decades. This nest fly dramatically reduces nestling survival of endemic Darwin's finches, and in some years, can cause up to 100% mortality (Koop et al., 2013, O'Connor et al., 2014). Compared to non-urban areas, finches have higher fledging success in urban areas (Harvey et al., 2021), which is likely related to food availability (De León et al., 2018). Because food availability can alter the host's ability to defend themselves against parasites (Knutie 2020), urban areas could amplify or dampen host defense strategies against this parasitic fly.

This study examines the effect of urbanization on the interactions between Darwin's finches and invasive avian vampire flies. For our first objective, we investigated whether the effect of the flies on finch nestlings differed in an urban and non-urban area. We experimentally

117 removed vampire flies from or allowed for natural parasitism in the nests of small ground finches
118 (*Geospiza fuliginosa*; a species of Darwin's finch) in an urban (Puerto Baquerizo Moreno) and
119 non-urban area (Jardín de las Opuntias) on San Cristóbal Island, Galápagos. We then quantified
120 nestling morphometrics, blood loss, and survival in response to parasitism across treatments and
121 locations. Relative diets were compared between urban and non-urban nestlings using stable
122 isotope analyses of feces ($\delta^{13}C$, $\delta^{15}N$, and C:N). Stable isotope analyses of adult flies were also
123 conducted to determine whether nestling diet had lasting effects on the flies into adulthood.
124 Because urban birds have better access to resources, which could positively affect immune
125 development, we expect that urban finches will be more resistant to vampire flies than non-urban
126 birds. Urban nestlings with better access to resources might also be able to recover blood loss the
127 parasites more efficiently and thus be more tolerant to parasitism. However, because urban birds
128 prefer human junk food (De León et al., 2018), which lacks many nutritional qualities required
129 for an active immune system, urban nestlings might not be well defended against the parasite.
130 Spatial variation of urban parasite abundance was also examined to determine whether parasite
131 load had spatial structure within the town or was associated with various environmental features
132 (e.g., restaurants).
133 Our second objective examined the molecular mechanisms that underlie the defense
134 response to flies in urban and non-urban nestlings. We characterized gene expression in the
135 blood of ~8 day old nestlings across parasite treatments and locations. Gene expression is the
136 process in which gene information is turned into a functional product, which can affect
137 phenotypes. For example, nestlings might have more relative expression of genes in a pathway
138 that affects erythrocyte production, which could account for higher tolerance to flies. Co-
139 expression profiles were also used to compare gene expression with organismal traits in the
140 nestlings. If urban nestlings are better defended against flies, we expect to observe increased

expression of genes related to innate and adaptive resistance and/or tolerance to blood loss compared to non-urban nestlings. Consequently, urban parasitized nestlings are expected to have better health and survival as compared to non-urban parasitized nestlings. Our study is one of the first to provide insight into the mechanisms by which urbanization positively or negatively influences host-parasite interactions.

Methods

Study system

We conducted our study between February–May 2019 in an urban and non-urban area in the dry lowland climatic zone of San Cristóbal (557 km^2) in the Galápagos Islands. The urban area encompasses the only city on San Cristóbal Island, Puerto Baquerizo Moreno (hereon, urban area) (-0.9067715°, -89.6061678°). This capital city is the second largest city in the Galápagos archipelago with a human population of 7,199 (INEC, 2016) and measures 0.79 km^2 (~1.2 km by 0.62 km), which includes tourist and residential areas (Harvey et al., 2021). The urban area is almost entirely consumed by human infrastructure, which primarily consists of impermeable concrete or stone surfaces and human built structures, but also includes native plants, such as matazárno (*Piscidia cathagenensi*), Galápagos acacia (*Acacia rorudiana*), and prickly pear cactus (*Opuntia megasperma*), and ornamental non-native plants established by humans.

The non-urban area is in Jardín de las Opuntias (hereon, non-urban area), which is a protected Galápagos National Park site located eight km southeast of the urban area (-0.9491651°, -89.5528454°). 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 (Harvey et al., 2021). The non-urban location did not contain any unnatural, human-built impermeable surfaces, and includes native plants, such as matazárno, acacia, and cacti. The non-urban area receives very low human visitation due to the

difficult terrain; however, local residents occasionally transect through the location to access the beach.

Small ground finches commonly nest in both the urban and non-urban area, generally between February–May (Harvey et al., 2021). Finches build their nests in matazárno, acacia, and cacti in both locations, but urban finches occasionally nest in human built structures such as gutters and building signs. Finches use coarse grasses and small twigs to build the outer structure of the nest, and finer, softer grasses and plants to construct the inner layer and nest liner. In urban areas, finches frequently incorporate trash and human hair into the construction of their nests (Theodospoulos & Gotanda 2018, Harvey et al., 2021). San Cristóbal small ground finches produce clutches between 1-5 eggs and females incubate the eggs for around 15 days (Harvey et al., 2021). After hatching, nestlings fledge when they are 12-16 days old. Although females are primarily involved in parental care, both males and females feed nestlings via regurgitation.

Nest parasite manipulation

Both field sites were searched daily or every other day for evidence of nest-building activity by small ground finches. Once a nest was located, it was checked every other day until eggs were laid in the nest. To check the nests, we used a small camera (Contour LLC), attached to an extendable pole, which transmitted video (via Bluetooth) to an iPhone.

Since avian vampire flies can lay their eggs during the finches' egg incubation stage, the nests were assigned to a control (naturally parasitized) or experimental (parasites removed) treatment after a full clutch of finch eggs was laid. We applied a 1% solution of controlled-release permethrin (Permacap: BASF Pest Control Solutions) to experimental nests and water to control nests. Permethrin has been used extensively by Galápagos researchers to experimentally remove vampire flies from nests (Fessl et al., 2006; Koop et al. 2013a, 2013b; Knutie et al.,

2014; Kleindorfer & Dudaniec, 2016; Knutie et al., 2016; McNew et al., 2019; Addesso et al., 2020) and is approved for use by the Galápagos National Park. We treated nests twice with their respective treatments: 1) during the egg stage, and 2) when nestlings hatched. During the first treatment, 3 mL of treatment was injected beneath the nest liner with a sterile blunt syringe. During the second treatment, the nest liner and nestlings were removed from the nest and the treatment was applied by spraying the treatment (10 times) into the base of the nest with a travel-sized spray bottle. The treatments were applied below the nest liner to ensure that the eggs and nestlings did not directly contact the treatment. While hatchlings were outside the nest during nest treatment, they were checked for signs of vampire fly infestation (black or enlarged nares, blood on legs, wings, or feather pores). The nest liner and nestlings were then returned to the nest. Julian hatch day and a GPS coordinate were recorded for each nest.

Nestling health and sample collection

We returned to the nest when nestlings were 7-8 days old to measure their body mass (to the nearest 0.1 g) with a portable digital scale balance (Ohaus CL2000) and morphometrics, such as tarsus, bill length, bill width, and bill depth (to the 0.01 mm) with analog dial calipers from Avinet. During this visit we opportunistically collected fecal samples from the nestlings. Briefly, a nestling was held approximately 10 cm over a sterile plastic weigh boat until it defecated (<10 seconds). We then transferred the fecal sac into a sterile 2 mL tube. The fecal sample was transferred to a 2mL tube and kept at 4°C in a portable insulin refrigerator until we returned from the field. Samples were stored in a -20°C freezer while in the Galápagos and then transferred to the University of Connecticut where they were stored at -80°C until processed for stable isotope analysis.

212 For up to three nestlings per nest, we also collected a small blood sample (<20 μL) from
213 the brachial vein using a 30-gauge sterile needle and heparinized capillary tube. Since nestlings
214 are being fed upon by a hematophagic ectoparasite, we wanted to collect the smallest blood
215 sample possible from each nestling. Therefore, we use a blood sample from each nestling within
216 a nest for a different assay. One sample was used to quantify hemoglobin levels (g/dL) with a
217 HemoCue® HB +201 portable analyzer (Hemocue America, USA) and one sample was used to
218 quantify glucose levels (mg/dL) with a glucometer (OneTouch, USA). The third sample of whole
219 blood was preserved in 180 μL of RNAlater; this preserved blood was kept at 4°C in a portable
220 insulin refrigerator until we returned from the field. The sample was then vortexed and stored at
221 4°C for 24 h before being placed in a -20°C freezer while in the Galápagos. The samples were
222 then transported to the University of Connecticut where they were stored at -80°C until extracted
223 for RNA sequencing.
224 We also banded nestlings with an individually numbered metal band and a unique
225 combination of three colored bands. When nestlings were approximately 12 days old, we
226 observed the nest with binoculars from a distance of approximately 5 m to prevent premature
227 fledging. Successful fledging was confirmed by identifying individual birds once they left the
228 nest.
229
230 *Quantifying parasite abundance*
231 After nestling birds fledged or died, the nest was collected and placed in a sealed, gallon-
232 sized plastic bag within two days of fledging. Nests were transported from the field and dissected
233 by hand to collect all stages of vampire flies present in the nest within 8 h. All larvae (1st, 2nd,
234 and 3rd instars), pupae, and pupal cases were identified and counted to determine total parasite
235 abundance for each nest. The length and width (0.01 mm) of up to ten pupae were haphazardly

236 measured with digital calipers. These measurements were used to calculate pupal volume ($V = \pi*[0.5*width]^2*length$). Third instar larvae were placed in ventilated 50 mL Falcon tubes with
237
238 their home nest material until pupation. Pupae were also placed in 50 mL Falcon tubes (without
239 material) until they eclosed and could be identified. Up to ten adult flies were collected and
240 preserved in 95% ethanol for stable isotope analysis (see below for methods).
241
242 *Stable isotope analyses*
243 We quantified $\delta^{13}C$ (^{13}C:^{12}C), $\delta^{15}N$ (^{15}N:^{14}N), and the carbon to nitrogen (C:N) ratio of
244 finch feces and adult fly bodies. $\delta^{13}C$ helps explain the differences in C4 vs. C3 plants consumed,
245 $\delta^{15}N$ helps explain the amount of dietary protein consumed (and infers trophic level), and C:N
246 helps explain the relative lipid consumption. Stable isotope analysis was used for flies to
247 examine whether nutritional differences were detectable in the vampire flies parasitizing the
248 finches. Feces were dried in an oven at 60°C for 24 h. Fly samples were rinsed three times in a
249 2:1 (vol/vol) chloroform/methanol mixture to remove surface oils, then rinsed with sterile water,
250 and dried at 60°C for 24 h. After drying, samples were ground to a fine powder using a mortar
251 and pestle. Up to 1 mg of each dried, homogenized sample was weighed into a tin capsule
252 (Costech Analytical Technologies, Inc., USA). Capsules were folded and placed into a 96-well
253 plate, then sent to the University of New Mexico Center for Stable Isotope Ratio Analysis
254 (SIRA). The samples were run as duplicates on a Thermo Delta V mass spectrometer connected
255 with a Costech 4010 Elemental Analyzer.
256
257 *Effect of location and landmarks on nesting and parasite abundance*
258 To explore whether location or landmarks (i.e., food markets, bakeries, benches and
259 restaurants) are associated with parasite abundance and fledging success, we conducted an

260 optimized hotspot analysis in ArcGIS 10.8.1 (ESRI 2020). First, a minimum convex polygon
261 (MCP) was created to serve as our study area for spatial analysis and included all landmarks
262 within the urban area. Nesting hotspots were then determined based exclusively on spatial
263 location of nests (i.e., clusters of finch nests rather than any other features on the landscape),
264 which resulted in a grid of cells that were characterized as hotspots or not within the MCP-
265 derived study area. To learn about the characteristics of these nests, the Near Tool was used to
266 identify and calculate the distance to the nearest landmark for all urban nest locations that were
267 within the nesting hotspots. Specifically, we were interested in all landmarks associated with
268 food, which was 190 records of 303 landmarks available. We then extracted the locations of
269 nests within hotspot locations and reviewed landmark types associated with these nests. Finally,
270 we conducted hotspot analyses based on parasite load (i.e., is there a hotspot of nest parasites?)
271 and also based on fledging (i.e., is there a hotspot of fledging success?). The hotspot analysis
272 uses the Getis Ord-Gi statistic (Getis & Ord, 1992), which determines spatial clusters with either
273 high or low values for the statistic (Fisher & Getis, 2010), corresponding to hot or cool spots,
274 respectively.
275
276 *RNA extractions, sequencing, and bioinformatics*
277 Total RNA was extracted from 100 µL of the whole blood and RNAlater solution using a
278 modified Tri-Reagent (Ambion, Invitrogen, USA) and Direct-zol RNA Miniprep Plus Kit (Zymo
279 Research, USA) protocol (Harvey & Knutie, 2022). The samples were incubated at room
280 temperature for 2 min and then centrifuged for 1 min at 8,000 x g to lightly pellet the blood.
281 Preservation fluid (RNAlater, Ambion, Invitrogen, USA) was pipetted off leaving no more than
282 ~15 µL of preservative and 500 µL of Tri-Reagent were added along with a sterile 5 mm
283 stainless steel bead (Thomson, USA) the samples were then vortexed for 30 seconds before

284 adding an additional 500 µL of Tri-Reagent. The sample was then vortexed for 10 min at room
285 temperature. The phase separation portion of the Tri-Reagent protocol was then followed, and
286 the upper aqueous phase (500 µL) was transferred to new microcentrifuge tubes. We then
287 followed the manufacturer's protocol for the Direct-zol RNA Kit beginning with the RNA
288 purification step. We eluted total RNA using 50 µL of RNA/DNA free water. We used a 4200
289 TapeStation and High Sensitivity RNA ScreenTape assays (Agilent, USA) to quantify total RNA
290 concentration (ng/µL) and RNA integrity numbers (RIN^e) (Schroeder et al., 2006). RNA extracts
291 were then stored at -80 °C until sequencing. The mean RNA concentration was 74.62 ng/µL
292 (range: 15.2-246.0) and mean RIN^e was 9.33 (range: 7.8-10), with all samples above the
293 minimum RIN^e cutoff (= 7.0) for successful sequencing.
294 A poly-A tail binding bead-based approach was used to reduce ribosomal RNA
295 contamination. First- and second-strand cDNA was synthesized using the Illumina TruSeq
296 Stranded mRNA Sample preparation kit and dual indexing was used to multiplex sequencing of
297 samples. Library quality was assessed on the Agilent Tapestation D1000 DNA High Sensitivity
298 assay and quantified using the Qubit 3.0 High Sensitivity dsDNA assay to ensure equimolar
299 pooling. A total of 42 libraries were sequenced across two (75bp PE) Illumina NextSeq500 High
300 Output sequencing runs.
301 Quality control was applied to paired-end libraries via Trimmomatic (v.0.39) (minimum
302 quality score 20 and minimum length 45bp) (Bolger et al., 2014). The trimmed reads were
303 aligned to the reference genome, *Geospiza fortis* (GeoFor_1.0, INSDC Assembly
304 GCA_000277835.1, Jul 2012), utilizing HISAT2 (v.2.2.1) (Kim et al., 2015). Read counts were
305 extracted from each alignment file via HTSeq (v.0.13.5) with the published reference annotation
306 (Anders et al., 2015).

307 Read counts and corresponding treatment and location assignments were imported into
308 RStudio (Bioconductor), and DESeq2 (v.1.32.0) was used to identify differentially expressed
309 genes among nestling groups (Soneson et al., 2015; Love et al., 2014). The factorial design with
310 an interaction term (~location + treatment + location:treatment) compared urban and non-urban
311 locations and parasite treatments (Fig. S2). *P*-values derived from the Wald test were corrected
312 for multiple testing using the Benjamini and Hochberg method (Love et al., 2014). We selected
313 genes with an adjusted *P*-value less than 0.1. Initial functional annotations were imported via
314 biomaRt (v.2.48.3) with Ensembl (bTaeGut1_v1.p) (Durinck et al., 2009). An enrichment and
315 depletion (two-sided hypergeometric test) *immune system process* Gene Ontology (GO)
316 enrichment analysis was performed on differentially expressed genes of sham-fumigated urban
317 and non-urban nestlings to predict potential immune function pathways. *Taeniopygia guttata*
318 (zebra finch) GO annotations were sourced from UniProt GOA and referenced within ClueGO
319 (v.2.5.9) and CluePedia (v.1.5.9), plug-ins for Cytoscape (v.3.8.2) (Shannon et al., 2003; Bindea
320 et al., 2009; Bindea et al., 2013). Terms with a *P*-value < 0.1 were considered significantly
321 enriched.
322 A co-expression analysis was conducted using WGCNA (v.1.71) in RStudio (Langfelder
323 & Horvath 2008). Binary and quantitative trait information was imported along with normalized
324 RNA-Seq counts. A soft-thresholding power of six was chosen based on the criterion of
325 approximate scale-free topology and sample size, and eigengene significance was calculated for
326 each module. Within Cytoscape, a ClueGO network (referencing zebra finch) was constructed to
327 visualize key drivers in the context of immune response.
328
329 *Statistical analyses*

330 Statistical analyses on field data (i.e., non-gene expression data) were conducted in R
331 (2021, v.1.4.1103) and figures were created in Prism (2021, v.9.2.0). All dependent variables
332 were tested for normality using Shapiro-Wilks tests. If variables did not pass normality ($P >$
333 0.05), they were $\log_{10}$ transformed to pass normality, as denoted below. Because mass and tarsus
334 were highly correlated ($R^2 = 0.74$, $P < 0.0001$), we calculated the scaled mass index, which is a
335 standardized metric of body condition (Peig & Green, 2009). Bill surface area was also
336 calculated from bill length, width, and depth using a modified equation for the surface area of a
337 cone (LaBarbera et al., 2017).
338 Linear mixed effects models (LMMs) with nest as a random effect were used to analyze
339 the effect of location on parasite size, effect of parasite intensity on parasite size, and effect of
340 Julian hatch day on parasite size. LMMs were used to determine the effect of location, treatment,
341 and their interaction on scaled mass index, bill shape, hemoglobin levels, and glucose levels,
342 with nest as a random effect. Nestling age (i.e., days old) and Julian hatch day were included as
343 covariates when they contributed significantly ($P < 0.05$) to the model (age only: bill shape;
344 Julian hatch day only: glucose; neither: scaled mass index, hemoglobin levels). LMMs were also
345 used to determine the effect of location and sample type (nestling feces vs. fly) on $\delta^{15}N$, $\delta^{14}C$,
346 and C:N, with sample replicate as a random effect.
347 Generalized linear models (GLMs) were used to analyze the effect of location on parasite
348 abundance (Poisson), the effect of location on Julian hatch day (log transformed; Gaussian), the
349 effect of location and Julian hatch day on brood size (log transformed; Gaussian), and effect of
350 Julian hatch day on parasite abundance (Poisson). GLMs were also used to determine the effect
351 of location, treatment, and their interaction on fledging age for the nest (log transformed;
352 Gaussian). A GLM with binomial errors for proportional data (i.e., a logistic regression) was

353 used to determine the effect of location, treatment, and their interaction on fledging success with
354 Julian hatch day as a covariate.
355 Analyses were conducted with the lmer function (LMMs) and glm function (GLMs)
356 using the lme4 package in R (Bates et al., 2015). Probability values were calculated using log-
357 likelihood ratio tests using the Anova function in the car package (Fox & Weisberg 2019). For
358 one-way and two-way ANOVAs, we used type II and type III sum of squares, respectively.
359

360 **Results**

361 *Effect of parasite treatment and urbanization on parasite load*

362 Urban and non-urban finches initiated breeding in early February 2019. The first urban
363 nests hatched approximately seven days before the first non-urban nest. Although Julian hatch
364 day was, on average, earlier for urban nests (mean ± SE: 86.24 ± 3.99 days) compared to non-
365 urban nests (94.17 ± 4.07 days), Julian hatch day did not differ significantly between locations
366 ($\chi^2 = 2.23$, $P = 0.14$). Furthermore, location, Julian hatch day, and their interaction did not
367 significantly affect brood size (urban: 2.66 ± 0.18 nestlings, $n = 38$ nests; non-urban: 2.87 ± 0.17,
368 $n = 30$ nests; location: $\chi^2 = 0.36$, $P = 0.55$; hatch day: $\chi^2 = 0.77$, $P = 0.38$; interaction: $\chi^2 = 1.09$,
369 $P = 0.30$). Eight nests were classified as depredated, either due to direct evidence of depredation
370 (e.g., body parts found) or because they were likely depredated (e.g., all nestlings disappeared
371 over a short period of time and prior to the typical fledging window). Three non-urban and five
372 urban nests were depredated, which were both permethrin-treated (three nests) and water-treated
373 (five nests).
374 The experimental treatment of nests with a mild insecticide was nearly 100% effective at
375 removing parasites (Fig. 1A; non-urban: $n = 13$ nests, urban: $n = 16$ nests); only one permethrin-
376 treated urban nest had eight parasites after the treatment. Since one permethrin-treated nest was

377 parasitized, nests from this treatment will be referred to as "fumigated". Water-treated nests
378 (hereafter, "sham-fumigated") were naturally parasitized by 27.00 (± 5.20) parasites in the non-
379 urban area (n = 17 nests) and 15.95 (± 3.15) parasites in the urban area (Fig. 1A; n = 21 nests).
380 One water-treated nest disappeared due to predation after the nestlings were banded and
381 therefore, we could not quantify parasitism. Fourteen of 17 (82.35%) non-urban nests and 17 of
382 21 (80.95%) urban nests contained at least one parasite. Within the sham-fumigated treatment,
383 non-urban nests had significantly fewer parasites than urban nests ($\chi^2 = 54.49$, $P < 0.0001$).
384 Julian hatch day did not significantly predict nest parasite abundance ($\chi^2 = 1.58$, $P = 0.21$).
385 The volume (i.e., size) of 93 pupae from 13 urban nests and 90 pupae from 14 non-urban
386 nests was calculated for comparison. Parasite size, which is a measure of virulence in
387 ectoparasites, was larger in urban nests compared to non-urban nests (Fig. 1B; $\chi^2 = 5.85$, $P =$
388 0.02). Urban parasites were, on average, 26% larger than non-urban parasites (urban: 100.30 ±
389 7.62 mm^3, non-urban: 81.61 ± 9.29 mm^3). This difference was not related to competition among
390 parasites within the nest because parasite intensity was not related to parasite size ($\chi^2 = 0.01$, $P =$
391 0.97). Rather, the nest hatch day predicted parasite size, with earlier nests having larger parasites
392 compared to later nesters (Fig. 1C; $\chi^2 = 11.01$, $P < 0.001$).
393
394 *Effect of location and landmarks on nesting and parasite abundance*
395 Of all urban nests (sham-fumigated and fumigated), one nesting hotspot was identified
396 within the urban area (n = 37 total nests; n =19 hotspot nests), which was at the airport (Fig. S1).
397 The hotspot grid resulted in 14 cells (78 m x 78 m) with 90% confidence of being a hotspot, with
398 Z-scores ranging between 2.90 and 6.02. The remaining cells in the study area were not
399 identified as significant nesting hot- or cool-spots. Of the 19 nests within the nesting hotspot,
400 seven contained parasites, and all but one of these was located near a bench. Hotspots were not

401 identified for fledging success, nor did we identify parasite hotspots among sham-fumigated
402 nests only.
403
404 *Effect of parasitism and urbanization on nestling health*
405 Nestlings from sham-fumigated nests had, on average, lower body condition (i.e., scaled
406 mass index) than nestlings from fumigated nests (Table S1; $\chi^2 = 4.01$, $P = 0.045$). Location and
407 the interaction between location and treatment did not significantly affect body condition
408 (location: $\chi^2 = 0.36$, $P = 0.55$; interaction: $\chi^2 = 2.20$, $P = 0.14$). Treatment and location did not
409 significantly affect bill surface area (location: $\chi^2 = 0.13$, $P = 0.72$; treatment: $\chi^2 = 0.71$, $P = 0.40$).
410 The interaction between treatment and location had a marginally non-significant effect on bill
411 surface area ($\chi^2 = 3.09$, $P = 0.08$); fumigated nestlings from non-urban areas had larger bill
412 surface area than nestlings from urban areas but the bill surface area for sham-fumigated
413 nestlings from urban and non-urban nests did not differ significantly. Most nestlings from non-
414 urban, sham-fumigated nests died before they could be measured; only 13 non-urban nestlings
415 from six sham-fumigated nests survived to be measured.
416 Location alone did not significantly affect nestling hemoglobin levels (Fig. 2; Table S1;
417 $\chi^2 = 1.06$, $P = 0.30$). Overall, sham-fumigated nestlings had lower hemoglobin levels than
418 fumigated nestlings ($\chi^2 = 18.82$, $P < 0.0001$). The interaction between treatment and location
419 affected hemoglobin levels ($\chi^2 = 6.16$, $P = 0.01$), with nestlings from sham-fumigated nests in
420 the non-urban area having, on average, 32% lower levels than nestlings from the other treatments
421 (i.e., sham-fumigated urban, fumigated urban, and fumigated non-urban nestlings).
422 Overall, nestlings from fumigated nests had higher glucose levels than nestlings from
423 sham-fumigated nests (Table S1; $\chi^2 = 9.75$, $P = 0.002$) and non-urban nestlings had higher
424 glucose levels than urban nestlings ($\chi^2 = 6.87$, $P = 0.009$). Additionally, the interaction between

425 treatment and location had an effect on nestling glucose levels ($\chi^2 = 9.34$, $P = 0.002$), with urban
426 nestlings from fumigated and sham-fumigated nests maintaining similar levels but non-urban
427 nestlings from fumigated nests having higher levels than sham-fumigated nestlings. Nestlings
428 from sham-fumigated nests in the non-urban area had, on average, 26% lower glucose levels than
429 nestlings from the other treatments (i.e., sham-fumigated urban, fumigated urban, and fumigated
430 non-urban nestlings).

431 Fledging success of urban and non-urban nestlings did not differ significantly ($\chi^2 = 0.67$,
432 $P = 0.41$), but overall, parasitism reduced fledging success (Fig. 1A; Table S1; $\chi^2 = 44.30$, $P <$
433 0.0001). The interaction between treatment and location affected fledging success ($\chi^2 = 8.28$, $P =$
434 0.004); survival of fumigated nestlings did not differ between locations (urban: 85%, non-urban
435 78%) but survival of sham-fumigated nestlings was lower in non-urban areas (7%), compared to
436 urban areas (48%). Age at fledging did not differ significantly between treatments ($\chi^2 = 0.20$, $P =$
437 0.66) but differed between locations ($\chi^2 = 3.71$, $P = 0.05$); urban nestlings left the nest
438 approximately one day later than non-urban nestlings. The interaction between treatment and
439 location did not significantly affect fledging age ($\chi^2 = 0.10$, $P = 0.75$).

440

441 *Effect of urbanization on finch and parasite diet*

442 Flies were enriched in $\delta^{15}N$ compared to nestling feces ($\chi^2 = 164.94$, $P < 0.0001$) and all
443 urban samples were enriched in $\delta^{15}N$ compared to non-urban samples ($\chi^2 = 108.13$, $P < 0.0001$;
444 Fig. 3A). However, the interaction between sample type and location did not significantly affect
445 $\delta^{15}N$ ($\chi^2 = 0.00$, $P = 0.99$). Nest parasite abundance correlated negatively with $\delta^{15}N$ values ($\chi^2 =$
446 5.69, $P = 0.02$; Fig. 3B). Nestling feces were enriched in $\delta^{13}C$ compared to flies ($\chi^2 = 11.21$, $P =$
447 0.0008). However, location or the interaction between sample type and location did not affect
448 $\delta^{13}C$ (location: $X^2 = 0.43$, $P = 0.51$; interaction: $\chi^2 = 1.16$, $P = 0.28$). Nestling feces had a higher

449 carbon to nitrogen ratio (C:N), compared to flies ($\chi^2 = 21.60$, $P < 0.0001$). However, location and
450 the interaction between sample type and location did not affect C:N (location: $\chi^2 = 2.04$, $P =$
451 0.15; interaction: $\chi^2 = 0.74$, $P = 0.39$).
452
453 *Sequencing, Quality Control, and Alignment*
454 In total, 42 paired-end nestling libraries were constructed for nestlings from sham-
455 fumigated urban nests ($n = 11$), sham-fumigated non-urban nests ($n = 5$), fumigated urban nests
456 ($n = 14$), and fumigated non-urban nests ($n = 12$). The total paired-reads post-QC ranged
457 between 15.86 M and 42.16 M and alignment rates against the *Geospiza fortis* genome ranged
458 from 80.74% to 91.81% (Table S2).
459
460 *Differentially Expressed Genes*
461 A pairwise differential expression analysis observing two sites (urban and non-urban) and
462 two treatments (sham-fumigated and fumigated) with an interaction produced four gene-sets
463 (File S1). A total of 5,123 genes ($P\text{-}adj < 0.1$) were differentially expressed in sham-fumigated
464 nestlings across the urban (up-regulated) and non-urban (down-regulated) sites - of which, 2,521
465 were up-regulated and 2,602 were down-regulated. Altogether, 57 genes demonstrated strong
466 expression patterns (± 4-fold change), including frizzled class receptor 10 FZD10 (16.20-fold
467 change), a primary receptor for Wnt signaling, leucine rich repeat and Ig domain containing 3
468 LINGO3 (5.35-fold change), which has been shown to regulate mucosal tissue regeneration in
469 humans and promote wound healing, adenosine deaminase ADA (-8.17-fold change), associated
470 with hemolytic anemia, and hepatic leukemia factor HLF (-5.64-fold change), known to
471 influence the renewal of hematopoietic stem cells (Wang et al., 2016; Zullo et al., 2021; Chen &
472 Mitchell, 1994; Komorowska et al., 2017). In fumigated nestlings, only two genes were up-

473 regulated in the urban site, bisphosphoglycerate mutase BPGM (3.23-fold change), a regulator of
474 erythrocyte metabolism and hemoglobin in red blood cells, and zinc finger protein GLIS1 (3.40-
475 fold change), which has been significantly associated with bill length (Xu et al., 2020;
476 Lundregan et al., 2018). There were no significantly down-regulated genes in fumigated urban
477 nestlings. When comparing sham-fumigated vs. fumigated nestlings in the urban site, a total of
478 768 genes were differentially expressed - with 570 being up-regulated in sham-fumigated
479 nestlings, and 198 down-regulated. The vast majority of genes showed moderate fold-change
480 patterns, with only three exhibiting strong expression patterns: inorganic pyrophosphate transport
481 regulator ANKH (4.43-fold change), prokineticin 2 PROK2 (4.38-fold change) and plexin A2
482 PLXNA2 (-4.08-fold change). By comparison, the non-urban site yielded 7,064 genes, 3,709
483 expressed in sham-fumigated nestlings, and 3,355 in fumigated nestlings. Of those, 314
484 displayed strong expression patterns, including assembly factor for spindle microtubules ASPM
485 (7.94-fold change), which has an apparent role in neurogenesis and neuronal development (Nam
486 et al., 2010) (Fig. S3-S4).
487
488 *Gene enrichment related to host defense mechanisms*
489 Gene enrichment of sham-fumigated nestlings from the urban and non-urban location
490 was compared to identify potential host defense mechanisms to explain the expression patterns
491 observed. Analysis of enriched *immune system process* Gene Ontology (GO) terms revealed 21
492 significant ($P < 0.05$) up-regulated terms (urban) and 12 significant down-regulated terms (non-
493 urban) categorized by resistance (adaptive and innate) and tolerance (File S2).
494 First, we examined enrichment patterns related to *adaptive immunological resistance*
495 (Fig. 4A). The sham-fumigated urban nestlings exhibited strong enrichment of lymphocyte
496 differentiation ($P < 0.02$) and T-cell pathways related to regulation of CD4-positive, alpha-beta T

497 cell activation ($P < 0.01$) and natural killer T-cell differentiation ($P < 0.05$). By comparison,
498 sham-fumigated non-urban nestlings yielded 21 significant adaptive immunity terms. Of those,
499 ten terms were related to Ig antibodies, including positive regulation of isotype switching to IgG
500 isotypes ($P < 0.001$) and somatic hypermutation of immunoglobulin genes ($P < 0.001$). The
501 other 11 terms were related to B cell proliferation ($P < 0.01$) and activation ($P < 0.05$), leukocyte
502 differentiation ($P < 0.04$) and proliferation ($P < 0.05$), regulation of antigen receptor-mediated
503 signaling pathways ($P < 0.04$), and germinal center formation ($P < 0.01$).
504 We next examined enrichment patterns related to *innate immunological resistance* (Fig.
505 4B). Sham-fumigated urban nestlings were enriched for both type I interferon mediated signaling
506 ($P < 0.03$) and toll-like receptor 9 signaling ($P < 0.05$). Among the sham-fumigated non-urban
507 nestlings, only negative regulation of innate immune response ($P < 0.07$) was enriched.
508 Finally, we examined enrichment related to *tolerance* (Fig. 4C). Sham-fumigated urban
509 nestlings had three significantly enriched terms specific to red blood cells, including erythrocyte
510 differentiation ($P < 0.001$) and development ($P < 0.001$), as well as enucleate erythrocyte
511 differentiation ($P < 0.01$). Among non-urban nestlings, regulation of megakaryocyte
512 differentiation yielded a *P*-value less than 0.07.
513
514 *Co-expression Analysis*
515 A differential co-expression analysis was conducted to provide novel insights on other
516 traits that may explain gene expression patterns in nestling finches. Correlations in transcript
517 levels across 12 traits (urban, sham-fumigated, hemoglobin concentration, glucose concentration,
518 survival ("alive"), deltaC, deltaN, C:N, days old, number of parasites in nest, bill surface area,
519 and scaled mass index) introduced 44 modules (File S3; Fig. S5-S8). Nestling survival ("alive"
520 trait) presented the most significant correlations across the gene set, as illustrated by module A (*r*

521 = 0.67, $P < 0.0001$) and module B ($r = -0.82$, $P < 0.0001$) respectively (Fig. 5). From the 2,338
522 total genes included in module A, 43 yielded 19 significantly enriched *immune system process*
523 terms related to T-cell activation, lymphocyte differentiation, and negative regulation of
524 hemopoiesis. Comparatively, 55 of the 3,422 genes in module B activated 21 immune pathways
525 related to somatic hypermutation of immunoglobulin genes, leukocyte mediated cytotoxicity and
526 interestingly, erythrocyte differentiation.

527 Apart from mortality, other traits were strongly correlated with module A and B.
528 Specifically, module A was negatively correlated with parasitism ($r = -0.59$, $P < 0.0001$) and
529 nest parasite abundance ($r = -0.74$, $P < 0.0001$), and positively correlated with hemoglobin level
530 ($r = 0.66$, $P < 0.0001$), glucose level ($r = 0.48$, $P < 0.001$), and deltaC ($r = 0.32$, $P < 0.04$).
531 Specifically, module B was positively correlated with parasitism ($r = 0.57$, $P < 0.0001$) and nest
532 parasite abundance ($r = 0.62$, $P < 0.0001$), and negatively correlated with hemoglobin level ($r =$
533 -0.52, $P < 0.0004$), glucose level ($r = -0.55$, $P < 0.0002$), deltaC ($r = -0.34$, $P < 0.03$) and scaled
534 mass ($r = -0.45$, $P < 0.003$). By comparison, although there were no notable correlations to
535 urbanization in the described modules, the enriched immune pathways better explain patterns of
536 expression observed in urban and non-urban sham-fumigated finches.

537

538 **Discussion**

539 Our study found that urban living partially rescues nestling small ground finches from the
540 lethal effects of parasitism by invasive avian vampire flies. Survival of sham-fumigated nestlings
541 did not differ significantly, which suggests that urbanization alone does not affect fledging
542 success. Urban nests had significantly fewer parasites than non-urban nests, but even highly
543 parasitized urban nestlings survived. These results indicate that urban nestlings have effective
544 resistance and tolerance mechanisms to deal with avian vampire flies. Differences in diet

545 (measured using stable isotopes) between locations could explain why urban nestlings are less
546 affected by parasites, but these results are correlative. Gene expression analyses revealed that
547 resistance and tolerance mechanisms might underlie differences in parasite effects. Sham-
548 fumigated urban nestlings differentially expressed genes within pathways associated with red
549 blood cell production (tolerance) and interferon type 1 activity (innate immunological
550 resistance), compared to sham-fumigated non-urban nestlings. In contrast, sham-fumigated non-
551 urban nestlings differentially expressed genes within pathways associated with immunoglobulin
552 production (adaptive immunological resistance). Both urban and non-urban nestlings expressed
553 different pathways related to T- and B-cell responses (adaptive immunological resistance).
554 However, urban nestlings also expressed pathways to downregulate T-cell production, which
555 might explain why immunoglobulin production genes were less expressed compared to non-
556 urban nestlings. The gene expression results suggest that non-urban nestlings are investing in
557 adaptive immunity, but that this type of response is not an effective defense mechanism against
558 the parasite since few nestlings survived.

559 Overall, our field data suggest that the urban finch population is investing in resistance
560 over tolerance because urban nests had 40% fewer parasites and higher nestling survival than
561 non-urban nests. Gene expression profiles showed that urban nestlings differentially express
562 innate immune genes associated with pro-inflammatory cytokines, specifically type-1 interferons
563 (IFNs), compared to non-urban nestlings. When larval flies chew through the skin of their hosts,
564 effective inflammation by the host (thickening the skin and restricting blood flow) can prevent
565 ectoparasite feeding (reviewed in Owens et al., 2010). Although other avian urbanization studies
566 have not observed changes in IFNs, they have found other enhanced innate immune responses
567 within urban populations. For example, Watson et al., (2017) observed overrepresented genes
568 involved in the secretion and receptor-binding of cytokines in urban great tits (*Parus major*)

569 compared to non-urban tits. Additionally, urban nestling black sparrowhawks (*Accipiter*
570 *melanoleucus*) had a stronger innate response to an immune challenge compared to non-urban
571 nestlings (Nwaogu et al., 2023). Although these studies were unable to link the heightened innate
572 immune response in urban nestlings to parasite resistance, they provide evidence that urban
573 living could confer an advantage against parasitism.

574 Most studies suggest that IFNs are largely involved in resistance to viruses (Katze et al.,
575 2002; Fensterl & Sen, 2009). An experimental study (e.g., with avian vampire fly-specific
576 vaccination challenges) is still needed to determine whether IFNs are specifically involved in the
577 inflammatory response to avian vampire flies because it is possible that finches are actually
578 responding to a virus. For example, adult finches in Puerto Baquerizo Moreno, San Cristóbal
579 Island, are susceptible to infection by the invasive avian pox virus (Lynton-Jenkins et al., 2021).
580 A recent study suggests that pox-infected adult finches upregulate expression of interferon
581 pathways (McNew et al., 2022), which could explain the expression of IFN seen in urban sham-
582 fumigated nestlings. One interesting possibility is that infection by pox could also be conferring
583 resistance to avian vampire flies. This potential explanation requires further study but could
584 provide insight into the disease dynamics of co-infecting invasive parasites of Galápagos birds
585 (Wikelski et al., 2004).

586 Although sham-fumigated non-urban nestlings did not exhibit significantly enriched
587 innate immune pathways, they did differentially express genes involved in pathways related to
588 the adaptive immune response. Specifically, T-cell, B-cell, and Ig pathways were expressed, but
589 without successfully conferring resistance since almost all of the nestlings died. This
590 upregulation could be the final effort by the finches' immune system to deal with the parasite
591 before it becomes physiologically costly. In contrast, urban finch nestlings expressed more
592 adaptive immune pathways related to lymphocyte and T-cell differentiation, compared to non-

urban nestlings. One explanation for why urban nestlings had higher survival compared to non-urban nestlings is that resistance is heightened when the innate and adaptive immune system are activated simultaneously (Palm & Medzhitov, 2007).

Regardless of the mechanism, a central question is why urban finches are more resistant to avian vampire flies than non-urban finches? Immunological resistance can be conditionally dependent and only hosts in good condition might be able to resist parasites (Sheldon & Verhulst, 1996; Svensson et al., 1998; Lochmiller & Deerenberg, 2000; Sternberg et al., 2012; Cornet et al., 2014; Howick & Lazzaro, 2014; Knutie, 2020). In a native host-parasite system, eastern bluebirds (*Sialia sialis*) are both tolerant and resistant to nest flies and the investment in defenses depends on whether supplemental food is available (resistance) or not (tolerance; Knutie, 2020). Urban areas in the Galápagos provide a reliable source of human food for animals, including finches (De León et al., 2018). For example, some restaurants have outdoor dining, where food falls off tables and finches feed on the tables (Fig. S9). Our stable isotope results corroborate the idea that diet differs between urban and non-urban nestlings. In fact, higher $\delta^{15}N$ values suggest a diet rich in meat, such as chicken, beef, fish, which can be found at outdoor restaurants in town. Higher $\delta^{15}N$ values also correlated with lower parasite abundances, which supports the idea that diet might influence host defenses. For example, meat has higher protein concentrations than plants and insects and supplemented protein can increase the concentration of cellular immune cells (e.g., eosinophils, globule leukocytes and mast cells) (reviewed in Coop & Kyriazakis, 2001).

Some urban nests had 100% nestling survival despite high parasite abundances (up to 48 parasites), indicating that these urban nestlings are tolerant of parasites. Studies have demonstrated that low finch survival in response to vampire flies is likely related to exsanguination (i.e., high blood loss) (Koop et al., 2013; Knutie et al., 2016). One explanation

617 for increased tolerance is that urban nestlings have effective blood recovery when parasitized and
618 thus tolerate parasitism. This hypothesis is corroborated with our results that urban finches have
619 more hemoglobin (oxygenated blood) when parasitized compared to non-urban birds. Gene
620 expression profiles suggest that urban nestlings differentially expressed genes within pathways
621 associated with red blood cell production compared to non-urban nestlings when parasitized.
622 Furthermore, nestlings that survived had higher gene expression of blood cell production than
623 nestlings that died, which was observed across urban and non-urban locations. Galápagos
624 mockingbirds are also relatively tolerant of avian vampire flies (Knutie et al., 2016), but this
625 tolerance is lost during dry years with low food availability (McNew et al., 2019). The working
626 hypothesis is that mockingbirds are a larger-bodied species and that larger hosts might be more
627 tolerant of avian vampire flies (McNew & Clayton, 2018). However, our study suggests that
628 even smaller-bodied hosts can tolerate avian vampire flies, and there is likely an alternative
629 explanation. The hormone erythropoietin is produced primarily by the kidneys and works
630 together with iron to induce erythropoiesis, which is the production of red blood cells. One
631 explanation for the lack of tolerance in non-urban nestlings is that these nestlings are not
632 receiving sufficient amounts of iron or are not producing enough erythropoietin. Urban nestlings
633 have higher $\delta^{15}N$ values, which is likely because they are feeding on more iron-rich meat
634 products, than non-urban birds. Thus, this difference in diet could explain the increased tolerance
635 related to red blood cell recovery but requires further studies on iron and nestling endocrinology.
636 Finally, although non-urban nestlings did not exhibit any significantly enriched pathways related
637 to tolerance, we found that adenosine deaminase (ADA) was significantly up-regulated.
638 Interestingly, overexpression of this gene in red blood cells has been shown to cause hemolytic
639 anemia (Chen & Mitchell, 1994), which could be additionally responsible for non-urban finch
640 mortality.

641 Although survival of urban nestlings in response to parasites is higher than non-urban
642 nestlings, some nests still failed due to parasitism. Therefore, not all urban finches have effective
643 defenses against avian vampire flies. Our urban field site is environmentally heterogeneous with
644 nests found in areas of high tourist activity, residential areas, a naval base, and the airport. Our
645 spatial analysis did not find any distinct patterns for parasite abundance or fledging success.
646 Thus, host-parasite dynamics could be related to the small radius (i.e., territory) around the nest,
647 which might not contribute to a larger spatial pattern. Alternatively, human food type,
648 abundance, and reliability vary based on tourist activity, which can change weekly depending on
649 whether cruise ships or other major tours are present. This activity can affect whether restaurants
650 are open and how much outdoor dining occurs, which can influence food availability for finches.
651 Naïve hosts are thought to lack defenses against novel parasites, which can cause species
652 declines and extinctions (Daszak et al., 2000; Keesing et al., 2010; Atkinson & Lapointe, 2009).
653 Over the past decade, studies have found that invasive avian vampire flies can cause up to 100%
654 mortality in Darwin's finch species across islands in the Galápagos (O'Connor et al., 2010; Koop
655 et al., 2010; Koop et al., 2013; O'Connor et al., 2014; Kleindorfer & Dudaniec 2016; Knutie et
656 al., 2016; McNew & Clayton, 2018; Addesso et al., 2021). If humans cannot effectively control
657 the fly (e.g., Knutie et al., 2014) or host species do not evolve or develop defenses, some species
658 might even go extinct (Fessl et al., 2010; Koop et al., 2013b). However, our study provides
659 evidence that urban finches can be relatively well-defended against avian vampire flies. We are
660 not suggesting that the Galápagos Islands should be urbanized to increase defense against
661 parasites. Instead, the goal of our study is to demonstrate that some finches can defend
662 themselves against the flies through various mechanisms, which can provide insight into
663 management strategies (Ohmer et al., 2021). One next step is to determine whether urban finches
664 have evolved defenses against the flies, which could be explored with a common garden

experiment (Lindström, 1999; Slagsvold et al., 2002; Schluter & Gustafsson, 2006). To determine whether food availability is responsible for enhanced host defenses, researchers could manipulate food availability then quantify its effect on finch health and defenses to parasitism. One method could be to install feeders for adults, who would use the food to feed their nestlings (Knutie, 2020). However, this method could benefit invasive predators of finches, such as cats or rats in the area (Phillips et al., 2012; Gotanda, 2021). Alternatively, nestlings could be directly supplemented with food, which would help control the amount of food provided to the birds. Overall, the results of our study present a potentially positive future for Darwin's finches in the face of a virulent invasive parasite and inspires the next step to understanding this dynamic system.

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690
691 **Data Availability Statement:** Details of full analysis, including intermediate files and
692 supporting scripts, are publicly available at: https://gitlab.com/PlantGenomicsLab/galapagos-
693 finch-rna-seq. All raw data are available on FigShare (DOI: available upon acceptance), with
694 individual-level nestling data in Table S1, and sequences have been uploaded to GenBank
695 (BioProject accession number: PRJNA930453).
696
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702
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927

928 **Figure legends**

929 **Fig. 1. A)** The influence of urbanization on mean (±SE) nest parasite abundance (number of
930 parasites per nest) and % fledging success. Fledging success did not differ for fumigated nests in
931 the urban and non-urban area. In sham-fumigated nests, urban nests had on average, fewer
932 parasites and higher fledging success compared to non-urban nests. Each point represents an
933 individual nest and darker points indicate higher sample sizes. Diamonds represent the fumigated
934 treatment and squares represent the sham-fumigated treatment. **B)** Parasite (pupal) volume (mm^3)
935 was larger in the urban area compared to the non-urban area. Each point represents a mean (±
936 SE) volume for a nest. **C)** Parasite volume decreased throughout the nesting season in both
937 locations. Means and standard error bars are represented in panels A and B.

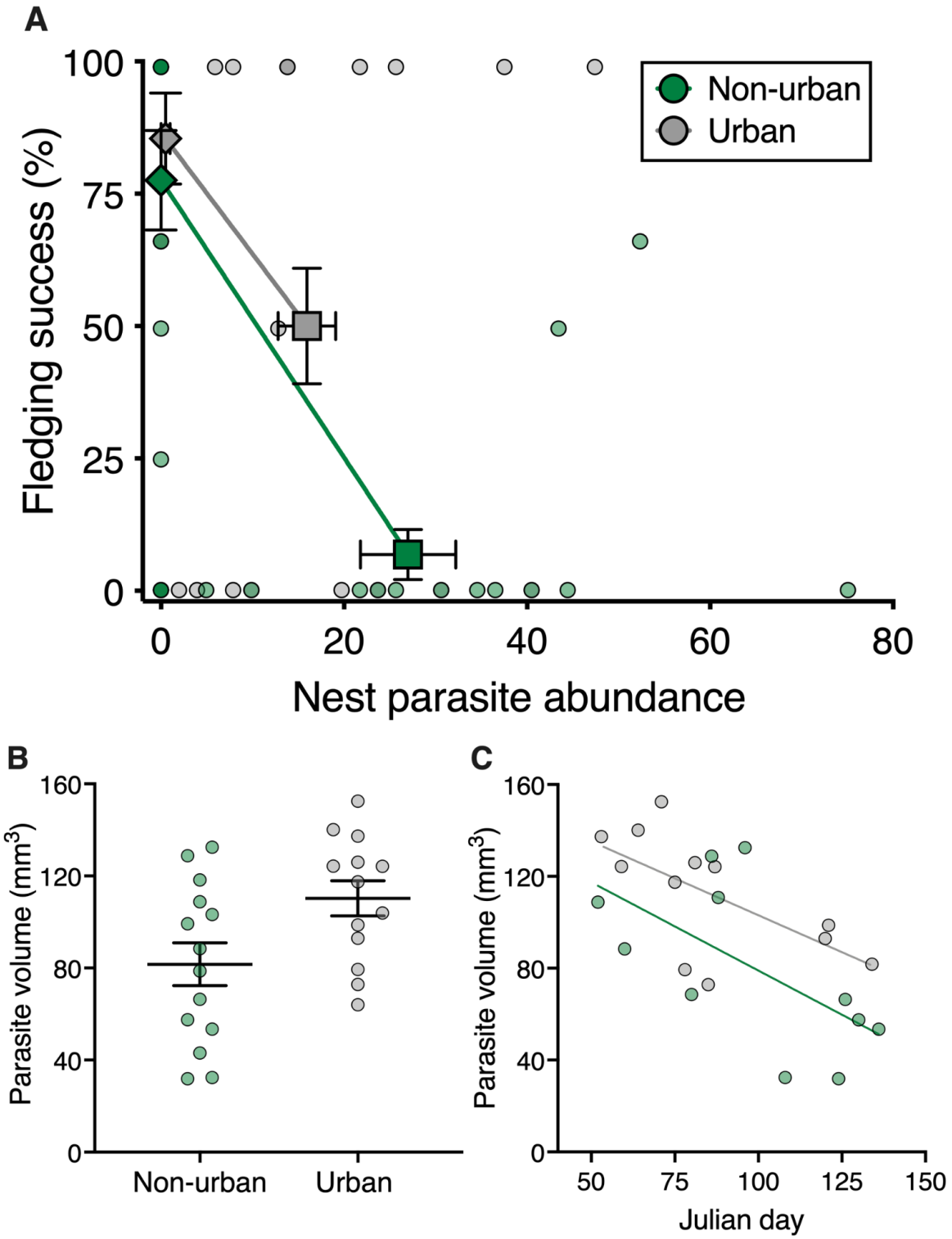
938 **Fig. 2.** Effect of parasitism and urbanization on mean (±SE) blood loss (hemoglobin) in nestling
939 finches. Nestlings from sham-fumigated nests had lower hemoglobin levels than nestlings from
940 fumigated nests. Non-urban nestlings from sham-fumigated nests had lower hemoglobin levels
941 than nestlings from the other treatments and locations.

942 **Fig. 3. A)** Mean (±SE) $\delta^{15}N$ and $\delta^{13}C$ values of nestlings and flies in the urban and non-urban
943 area. Urban individuals were enriched with $\delta^{15}N$ compared to non-urban individuals. Overall,
944 flies were enriched with $\delta^{15}N$ compared to nestlings and nestlings were enriched with $\delta^{13}C$
945 compared to flies. **B)** Nest parasite abundance (number of parasites per nest) was correlated
946 negatively with $\delta^{15}N$ values. Each point represents an individual.

947 **Fig. 4.** *Immune system process* Gene Ontology enrichment analysis of sham-fumigated urban
948 (up-regulated) and non-urban (down-regulated) differentially expressed genes utilizing ClueGO.
949 A) Adaptive Immune Response functional categories shown in purple were partitioned into
950 seven sub-categories: B Cell, Ig Antibodies, Leukocyte, Receptor, Structure, T Cell and Other.
951 (B) Innate Immune Response categories, shown in red, were divided into four sub-categories:

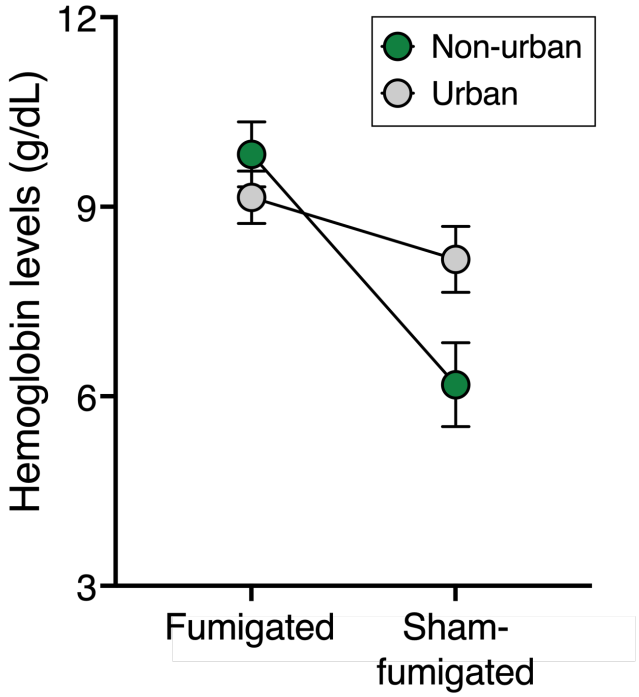
952 Lymphocyte, Pattern Recognition, Pro-Inflammatory and Other. (C) Tolerance categories, shown
953 in orange, were split into two sub-categories: Erythrocyte and Hematopoietic. Log10 (*P*-value)
954 significance of unique and shared terms is depicted a color saturation gradient (** = *P*-value <
955 0.05), and number of genes supporting each ontology term is represented by circle size.
956 **Fig. 5.** *Immune system process* Gene Ontology enrichment network of co-expression module A
957 (top) and B (bottom). WGCNA trait correlation is depicted on a scale from 0 (white) to 1 (red),
958 and negative trait correlation significance from 0 (white) to -1 (blue). WGCNA *P*-values are
959 shown in parentheses. The co-expressed genes are represented with ClueGO in pathway form.
960 The significance of enriched Gene Ontology terms is represented by the gray gradient. The size
961 of the circle represents the number of genes associated with the enriched term.
962

963 **Fig 1.**


A
Fledging success (%)
100
75
50
25
0
0
20
40
60
80
Nest parasite abundance
Non-urban
Urban
B
Parasite volume (mm3)
160
120
80
40
0
Non-urban
Urban
C
Parasite volume (mm3)
160
120
80
40
0
50
75
100
125
150
Julian day

964

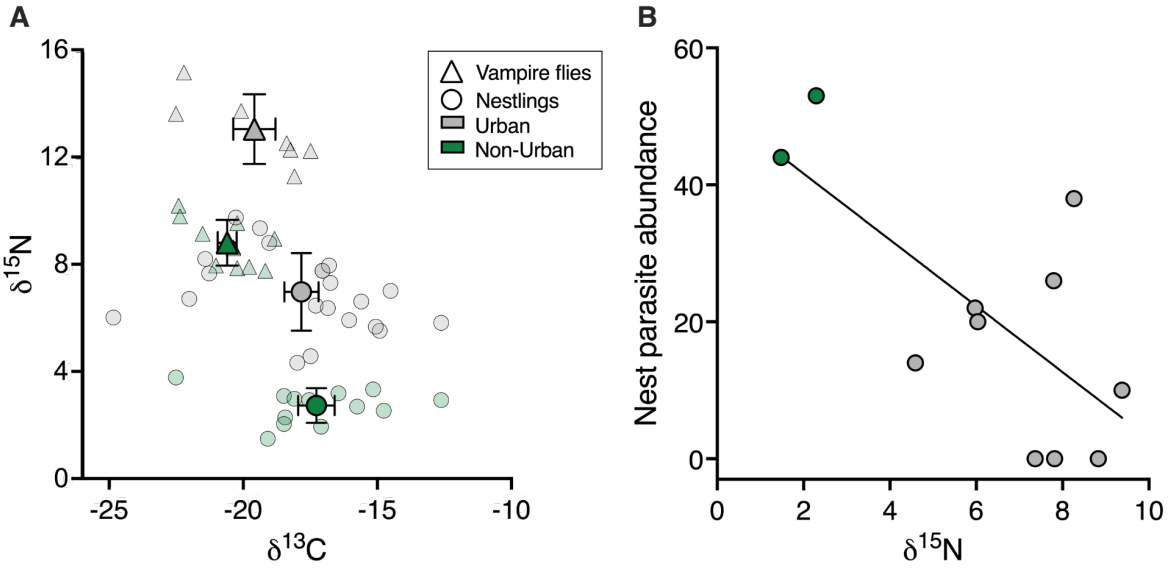
965 **Fig. 2.**



966

967

968 **Fig. 3.**


A
16
12
8
4
0
δ15N
-25
-20
-15
-10
δ13C
Vampire flies
Nestlings
Urban
Non-Urban
B
60
40
20
0
Nest parasite abundance
0
2
4
6
8
10
δ15N

969

Fig. 4.

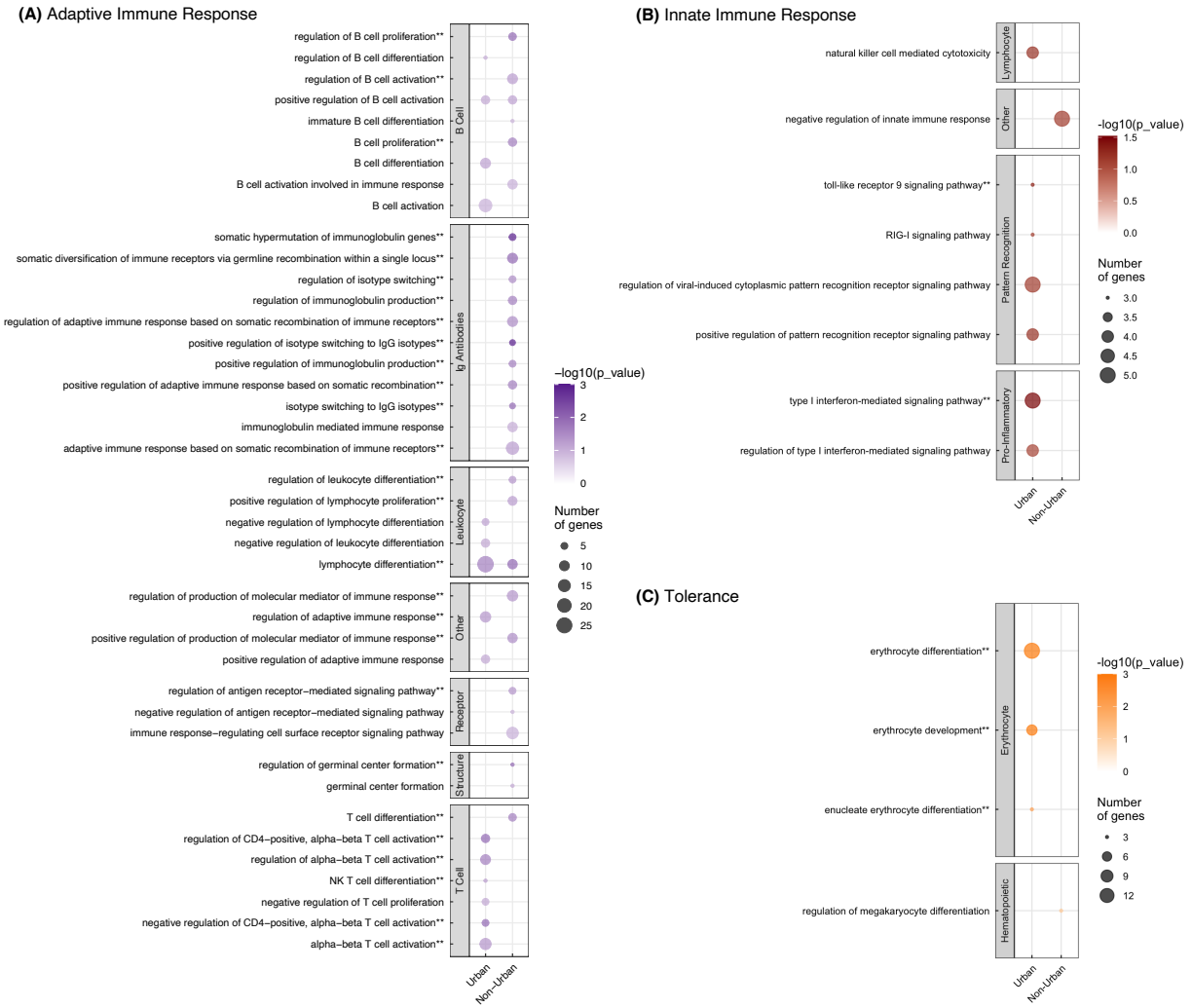
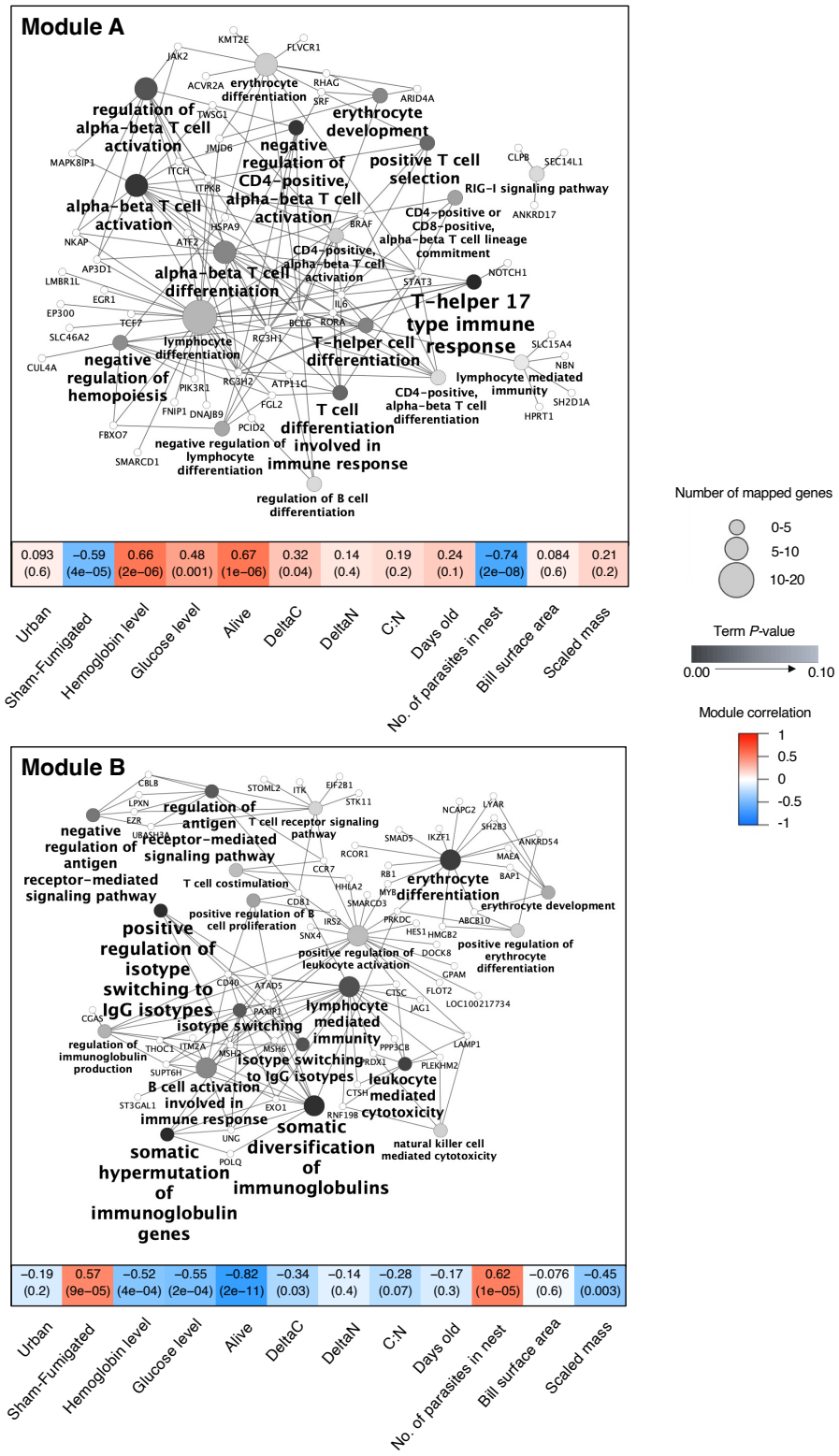

(A) Adaptive Immune Response
regulation of B cell proliferation**
regulation of B cell differentiation
regulation of B cell activation**
positive regulation of B cell activation
immature B cell differentiation
B cell proliferation**
B cell differentiation
B cell activation involved in immune response
B cell activation
B Cell
somatic hypermutation of immunoglobulin genes**
somatic diversification of immune receptors via germline recombination within a single locus**
regulation of isotype switching**
regulation of immunoglobulin production**
regulation of adaptive immune response based on somatic recombination of immune receptors**
positive regulation of isotype switching to IgG isotypes**
positive regulation of immunoglobulin production**
positive regulation of adaptive immune response based on somatic recombination**
isotype switching to IgG isotypes**
immunoglobulin mediated immune response
adaptive immune response based on somatic recombination of immune receptors**
Ig Antibodies
regulation of leukocyte differentiation**
positive regulation of lymphocyte proliferation**
negative regulation of lymphocyte differentiation
negative regulation of leukocyte differentiation
lymphocyte differentiation**
Leukocyte
regulation of production of molecular mediator of immune response**
regulation of adaptive immune response**
positive regulation of production of molecular mediator of immune response**
positive regulation of adaptive immune response
Other
regulation of antigen receptor−mediated signaling pathway**
negative regulation of antigen receptor−mediated signaling pathway
immune response−regulating cell surface receptor signaling pathway
Receptor
regulation of germinal center formation**
germinal center formation
Structure
T cell differentiation**
regulation of CD4−positive, alpha−beta T cell activation**
regulation of alpha−beta T cell activation**
NK T cell differentiation**
negative regulation of T cell proliferation
negative regulation of CD4−positive, alpha−beta T cell activation**
alpha−beta T cell activation**
T Cell
Urban
Non−Urban
−log10(p_value)
3
2
1
0
Number of genes
5
10
15
20
25
(B) Innate Immune Response
natural killer cell mediated cytotoxicity
Lymphocyte
negative regulation of innate immune response
Other
toll-like receptor 9 signaling pathway**
RIG-I signaling pathway
regulation of viral-induced cytoplasmic pattern recognition receptor signaling pathway
positive regulation of pattern recognition receptor signaling pathway
Pattern Recognition
type I interferon-mediated signaling pathway**
regulation of type I interferon-mediated signaling pathway
Pro-Inflammatory
Urban
Non-Urban
-log10(p_value)
1.5
1.0
0.5
0.0
Number of genes
3.0
3.5
4.0
4.5
5.0
(C) Tolerance
erythrocyte differentiation**
erythrocyte development**
enucleate erythrocyte differentiation**
Erythrocyte
regulation of megakaryocyte differentiation
Hematopoietic
Urban
Non-Urban
-log10(p_value)
3
2
1
0
Number of genes
3
6
9
12

Fig. 5


Module A
KMT2E
FLVCR1
JAK2
ACVR2A
erythrocyte differentiation
RHAG
SRF
ARID4A
regulation of alpha-beta T cell activation
TWSG1
erythrocyte development
negative regulation of CD4-positive, alpha-beta T cell activation
JMJD6
positive T cell selection
CLPB
SEC14L1
MAPK8IP1
ITCH
ITPKB
RIG-I signaling pathway
alpha-beta T cell activation
CD4-positive or CD8-positive, alpha-beta T cell lineage commitment
ANKRD17
HSPA9
BRAF
NKAP
ATF2
CD4-positive, alpha-beta T cell activation
AP3D1
alpha-beta T cell differentiation
NOTCH1
LMBR1L
STAT3
EGR1
IL6
T-helper 17 type immune response
EP300
TCF7
BCL6
RORA
SLC46A2
lymphocyte differentiation
RC3H1
T-helper cell differentiation
SLC15A4
CUL4A
NBN
negative regulation of hemopoiesis
RC3H2
ATP11C
lymphocyte mediated immunity
PIK3R1
CD4-positive, alpha-beta T cell differentiation
SH2D1A
FNIP1
DNAJB9
FGL2
T cell differentiation involved in immune response
HPRT1
FBXO7
PCID2
negative regulation of lymphocyte differentiation
SMARCD1
regulation of B cell differentiation
0.093 (0.6)
−0.59 (4e−05)
0.66 (2e−06)
0.48 (0.001)
0.67 (1e−06)
0.32 (0.04)
0.14 (0.4)
0.19 (0.2)
0.24 (0.1)
−0.74 (2e−08)
0.084 (0.6)
0.21 (0.2)
Urban
Sham-Fumigated
Hemoglobin level
Glucose level
Alive
DeltaC
DeltaN
C:N
Days old
No. of parasites in nest
Bill surface area
Scaled mass
Module B
CBLB
STOML2
ITK
EIF2B1
LPXN
regulation of antigen receptor-mediated signaling pathway
STK11
EZR
T cell receptor signaling pathway
NCAPG2
LYAR
negative regulation of antigen receptor-mediated signaling pathway
UBASH3A
SH2B3
SMAD5
IKZF1
ANKRD54
RCOR1
MAEA
CCR7
T cell costimulation
RB1
erythrocyte differentiation
BAP1
HHLA2
MYB
CD81
SMARCD3
erythrocyte development
positive regulation of B cell proliferation
PRKDC
ABCB10
IRS2
HES1
HMGB2
positive regulation of isotype switching to IgG isotypes
SNX4
positive regulation of leukocyte activation
DOCK8
positive regulation of erythrocyte differentiation
GPAM
CD40
ATAD5
FLOT2
CTSC
LOC100217734
JAG1
CGAS
PAXIP1
lymphocyte mediated immunity
isotype switching
LAMP1
regulation of immunoglobulin production
THOC1
ITM2A
MSH6
PPP3CB
MSH2
isotype switching to IgG isotypes
RDX1
PLEKHM2
SUPT6H
B cell activation involved in immune response
CTSH
leukocyte mediated cytotoxicity
ST3GAL1
EXO1
RNF19B
somatic diversification of immunoglobulins
natural killer cell mediated cytotoxicity
UNG
somatic hypermutation of immunoglobulin genes
POLQ
−0.19 (0.2)
0.57 (9e−05)
−0.52 (4e−04)
−0.55 (2e−04)
−0.82 (2e−11)
−0.34 (0.03)
−0.14 (0.4)
−0.28 (0.07)
−0.17 (0.3)
0.62 (1e−05)
−0.076 (0.6)
−0.45 (0.003)
Urban
Sham-Fumigated
Hemoglobin level
Glucose level
Alive
DeltaC
DeltaN
C:N
Days old
No. of parasites in nest
Bill surface area
Scaled mass
Number of mapped genes
0-5
5-10
10-20
Term P-value
0.00
0.10
Module correlation
1
0.5
0
-0.5
-1