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 fumigated nestlings from urban and non-urban nests did not differ significantly. However, sham-fumigated, non-urban nestlings lost more blood and few nestlings survived compared to urban nestlings. Stable isotopic values ($\delta^{15}N$) from urban nestling feces were higher than non-urban nestlings, suggesting that urban nestlings are consuming more protein. $\delta^{15}N$ 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 sham-fumigated non-urban nestlings. In contrast, sham-fumigated 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.

44 **Key words:** Darwin's finch, ecoimmunology, host defenses, gene expression, invasive parasites,
45 resistance, stable isotopes, tolerance, transcriptomics

Introduction

Emerging diseases are a major global threat to biodiversity (Daszak et al. 2000, Keesing et al. 2010). Naïve hosts who cannot effectively defend themselves against novel disease-causing parasites may risk population declines or even extinction (van Riper III and van Riper 1986, Frick et al. 2010). However, not all hosts are susceptible to introduced parasites. The fitness of some host species is clearly reduced, while the fitness of other hosts is relatively unaffected. Less affected hosts may alleviate parasite damage with defense mechanisms, such as tolerance and resistance (Read et al. 2008). Tolerance mechanisms, such as tissue repair or recovery of blood loss due to parasites, minimize the damage that parasites cause to the host without reducing parasite fitness (Medzhitov, Schneider, & Soares, 2012; Miller, White, & Boots, 2006; Råberg, Sim, & Read, 2007; Read, Graham, & Råberg, 2008). For example, parents from ectoparasite-infested nests reduce the cost of parasitism by feeding their offspring more than parents from fumigated nests (Christe, Richner, & Oppliger, 1996; Knutie et al., 2016; Tripet & Richner, 1997). Consequently, despite infestation, offspring do not suffer a high cost of parasitism because they are able to compensate for resources lost to the parasites. Alternatively, but not mutually exclusive, resistance, such as the immune response, minimizes the damage that parasites cause to the host by reducing parasite fitness (Read et al., 2008). The presence and effectiveness of host defenses to invasive parasites is highly variable among individuals and populations and can depend on how quickly they can evolve these responses or whether specific environmental factors are available to facilitate them (Feis et al. 2016, McNew et al. 2019).

Humans in urban environments can increase anthropogenic food availability and reliability for animals with the establishment of wild animal feeders or the incomplete disposal of human trash. Due to the high energetic cost of defense mechanisms, only hosts with sufficient food resources, such as in these urban areas, may be able to invest in defenses (Cornet, Bichet,

Larcombe, Faivre, & Sorci, 2014; Howick & Lazzaro, 2014; Lochmiller & Deerenberg, 2000; Sheldon & Verhulst, 1996; Sternberg et al., 2012; Svensson, Råberg, Koch, & Hasselquist, 1998; Knutie 2020). Extra nutrients obtained from human-derived food, such as protein, can directly increase the production of immune cells (Coop & Kyriazakis, 2001; Strandin, Babayan, & Forbes, 2018). Consequently, individuals with less effective defenses but better access to resources might be better equipped to resist the negative effects of parasites. Alternatively, human-supplemented food may be of lower nutritional quality, which could decrease the hosts' health (Catto et al. 2021) and therefore their ability to produce an effective immune response to parasites. Without the development or evolution of resistance and tolerance defenses, host population size can decrease or even go extinct, and this effect might be especially apparent in the context of human-influenced environments (van Riper III and van Riper 1986, Atkinson and 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 and Wachter 2011, Zhou et al. 2015, 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). The Galápagos Islands of Ecuador are relatively pristine but face an increasing rate of change 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 and Mena 2016). The introduction of novel parasites to the Galápagos is also relatively recent, such as the avian vampire fly (*Philornis downsi*). The adult fly was first collected in the Galápagos in 1964

(Causton et al. 2006), but the first sign of parasitism in nests was in 1997 (Fessl and Tebbich 2002). Adult flies are non-parasitic but lay their eggs in the nests of birds. Once hatched, fly larvae feed on the blood of nestling and brooding female birds (Fessl and Tebbich 2002, Koop et al. 2013). This fly dramatically reduces nestling survival of endemic Darwin's finches, and in some years, can cause up to 100% mortality of infested nestlings (Koop et al. 2013, O'Connor et al. 2014). However, some endemic Galapagos species, such as the Galapagos mockingbird (*Mimus parvulus*), are able to tolerate the effect of the fly because parents of parasitized nests feed their nestlings more than parents of non-parasitized nests to compensate for energy lost to the flies. Because urban areas offer more consistent food, though likely of lower quality, (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 removed vampire flies from or allowed for natural parasitism in the nests of small ground finches (*Geospiza fuliginosa*; a species of Darwin's finch) in an urban (Puerto Baquerizo Moreno) and non-urban area (Jardin de las Opuntias) on San Cristóbal Island, Galápagos. We then quantified nestling morphometrics, blood loss, and survival in response to parasitism across treatments and sites. Relative diets were compared between urban and non-urban nestlings using stable isotope analyses of feces ($\delta^{13}C$, $\delta^{15}N$, and C:N). Stable isotope analyses of adult flies were also conducted to determine whether nestling isotope values were reflected in the flies. Because urban birds have better access to resources, which could positively affect immune development, we expect that urban finches will be more resistant to vampire flies than non-urban birds. However, because urban birds prefer human-processed food (De León et al. 2018), which lacks many nutritional qualities required for an active immune system, urban nestlings might not be well-

118 defended against the parasite. Spatial variation of urban parasite abundance was also examined to
119 determine whether parasite load had spatial structure within the urban area or was associated
120 with various environmental features (e.g., restaurants).

121 Our second objective examined the molecular mechanisms that underlie the defense
122 response to flies in urban and non-urban nestlings. We characterized gene expression in the
123 blood of ~8 day old nestlings across parasite treatments and locations. Gene expression is the
124 process in which gene information is turned into a functional product, which can affect
125 phenotypes. For example, nestlings in urban areas may have more relative expression of genes in
126 a pathway that affects erythrocyte production, which could account for higher tolerance to flies.
127 Co-expression profiles were also used to compare gene expression with organismal traits of
128 nestlings. If urban nestlings are better defended against flies, we expect to observe increased
129 expression of genes related to innate and adaptive resistance and/or tolerance to blood loss
130 compared to non-urban nestlings. Consequently, urban, sham-fumigated nestlings are expected
131 to have better health and survival as compared to non-urban, sham-fumigated nestlings. Our
132 study is one of the first to provide insight into the mechanisms by which urbanization positively
133 or negatively influences host-parasite interactions.

134

135 **Methods**

136 *Study system*

137 We conducted our study between February–May 2019 in an urban and non-urban area in
138 the dry lowland climatic zone of San Cristóbal (557 km^2) in the Galápagos Islands. The urban
139 area encompasses the only city on San Cristóbal Island, Puerto Baquerizo Moreno (hereon, urban
140 area) (-0.9067715°, -89.6061678°). This capital city is the second largest city in the Galápagos
141 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 kilometers southeast of the urban area (-0.9491651°, -89.5528454°). Our non-urban study area measures 0.21 km^2 and covers 1.4 km of the main trail and 0.15 km to each side. The non-urban site does 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 site to access the beach.

Small ground finches on San Cristóbal 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 and Gotanda 2018, Harvey et al. 2021). 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. Observations were made primarily through binoculars to minimize nest disturbance. When adults were not at the nest, we used a small camera (Contour LLC), attached to an extendable pole, which transmitted video (via Bluetooth) to an iPhone.

Since vampire flies can lay their eggs during the finches' egg incubation stage, the nests were assigned to a control (hereon, sham-fumigated) or experimental (hereon, fumigated) 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 avian vampire flies from nests (Fessl et al. 2006, Koop et al. 2013a,b, Knutie et al. 2014, Kleindorfer and 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 permethrin or water 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 permethrin or water was applied by spraying (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 permethrin or water. While hatchlings were outside the nest during nest treatment, they were checked for signs of vampire fly infestation (black or enlarged nares, or blood on legs, wings, or feather pores). The nest liner and nestlings were then returned to the nest, Julian hatch day was recorded, and a GPS coordinate was taken for each nest.

190 *Nestling health and sample collection*

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

201 For up to three nestlings per nest, we also collected a small blood sample (<20 µL) from
202 the brachial vein using a 30-gauge sterile needle and heparinized capillary tube. Since nestlings
203 are being fed upon by a hematophagic ectoparasite, we wanted to collect the smallest blood
204 sample possible from each nestling. Therefore, we used a blood sample from each nestling
205 within a nest for a different assay. One sample was used to quantify hemoglobin levels (g/dL)
206 with a HemoCue® HB +201 portable analyzer (Hemocue America, USA). The second sample
207 was used to quantify glucose levels (mg/dL) with a glucometer (OneTouch, USA). The third
208 sample of whole blood was preserved in 180 µL of RNAlater; this preserved blood was kept at
209 4°C in a portable insulin refrigerator until we returned from the field. The sample was then
210 vortexed and stored at 4°C for 24 h, according to manufacturer's protocol, before being placed in
211 a -20°C freezer while in the Galápagos (up to two months). The samples were then transported
212 on ice packs (20 h) to the University of Connecticut where they were stored for two months at -
213 80°C until extracted for RNA sequencing.

We banded nestlings with an individually numbered metal band and a unique combination of three colored bands. When nestlings were approximately 12 days old, we observed the nest every two days with binoculars from a distance of approximately 5 m (to prevent premature fledging). Successful fledging was confirmed by identifying individual birds once they left the nest.

Quantifying parasite abundance

Within two days of all nestling birds either fledging or dying, the nest was collected and placed in a sealed, gallon-sized plastic bag. Nests were transported from the field and dissected by hand to collect all stages of vampire flies present in the nest within 8 h. All larvae (1st, 2nd, and 3rd instars), pupae, and pupal cases were identified and counted to determine total parasite abundance for each nest. The length and width (0.01 mm) of up to ten pupae were haphazardly 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 their home nest material until pupation. Pupae were also placed in 50 mL Falcon tubes (without material) until they eclosed. Once they eclosed, up to ten adult flies were collected and preserved in 95% ethanol for stable isotope analysis (see below for methods).

Stable isotope analyses

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 finch feces and adult fly bodies. $\delta^{13}C$ helps explain the differences in C4 vs. C3 plants consumed, $\delta^{15}N$ helps explain the amount of dietary protein consumed (and infers trophic level), and C:N helps explain the relative lipid consumption. Stable isotope analysis was used for flies to examine whether nutritional differences were detectable in the vampire flies parasitizing the

238 finches. Feces were dried in an oven at 60°C for 24 h. Fly samples were rinsed three times in a
239 2:1 (vol/vol) chloroform/methanol mixture to remove surface oils, then rinsed with sterile water,
240 and dried at 60°C for 24 h. After drying, samples were ground to a fine powder using a mortar
241 and pestle. Up to 1 mg of each dried, homogenized sample was weighed into a tin capsule
242 (Costech Analytical Technologies, Inc., USA). Capsules were folded, and placed into a 96-well
243 plate, then sent to the University of New Mexico Center for Stable Isotope Ratio Analysis
244 (SIRA). The samples were run as duplicates on a Thermo Delta V mass spectrometer connected
245 with a Costech 4010 Elemental Analyzer.
246
247 *Effect of location and landmarks on parasite abundance and fledging success*
248 To explore whether location or landmarks (i.e. food markets, bakeries, benches and
249 restaurants) are associated with parasite abundance and fledging success, we conducted an
250 optimized hotspot analysis in ArcGIS 10.8.1 (ESRI 2020). First, a minimum convex polygon
251 (MCP) was created to serve as our study area for spatial analysis and included all landmarks
252 within the urban area. Nesting hotspots were then determined based exclusively on spatial
253 location of nests (i.e., clusters of finch nests rather than any other features on the landscape),
254 which resulted in a grid of cells that were characterized as hotspots or not within the MCP-
255 derived study area. To learn about the characteristics of these nests, the Near Tool was used to
256 identify and calculate the distance to the nearest landmark for all urban nest locations that were
257 within the nesting hotspots. Specifically, we were interested in all landmarks associated with
258 food, which was 190 records of 303 landmarks available. We then extracted the locations of
259 nests within hotspot locations and reviewed landmark types associated with these nests. Finally,
260 we conducted hotspot analyses based on parasite load (i.e., is there a hotspot of nest flies?) and
261 also based on fledging (i.e., is there a hotspot of fledging success?). The hotspot analysis uses the

262 Getis Ord-Gi statistic (Getis and Ord 1992), which determines spatial clusters with either high or
263 low values for the statistic (Fisher and Getis 2010), corresponding to hot or cool spots,
264 respectively.
265
266 *RNA extractions, sequencing, and bioinformatics*
267 Total RNA was extracted from 20 µL of peripheral whole blood using a modified Tri-
268 Reagent (Ambion, Invitrogen, USA) and Direct-zol RNA Miniprep Plus Kit (Zymo Research,
269 USA) protocol (Harvey and Knutie 2022). The samples were incubated at room temperature for
270 2 min and then centrifuged for 1 min at 8,000 x g to lightly pellet the blood. Preservation fluid
271 (RNAlater, Ambion, Invitrogen, USA) was pipetted off leaving no more than ~15 µL of
272 preservative and 500 µL of Tri-Reagent were added along with a sterile 5 mm stainless steel
273 bead (Thomson, USA). The samples were then vortexed for 30 seconds before adding an
274 additional 500 µL of Tri-Reagent. The sample was then vortexed for 10 min at room
275 temperature. The phase separation portion of the Tri-Reagent protocol was then followed, and
276 the upper aqueous phase (500 µL) was transferred to new microcentrifuge tubes. We then
277 followed the manufacturer's protocol for the Direct-zol RNA Kit beginning with the RNA
278 purification step. We eluted total RNA using 50 µL of RNA/DNA free water. We used a 4200
279 TapeStation and High Sensitivity RNA ScreenTape assays (Agilent, USA) to quantify total RNA
280 concentration (ng/µL) and RNA integrity numbers (RIN^e) (Schroeder et al. 2006). RNA extracts
281 were then stored at -80 °C until sequencing. The mean RNA concentration was 74.62 ng/µL
282 (range: 15.2-246.0) and mean RIN^e was 9.33 (range: 7.8-10), with all samples above the
283 minimum RIN^e cutoff (= 7.0) for successful sequencing.
284 A poly-A tail binding bead-based approach was used to reduce ribosomal RNA
285 contamination. First- and second-strand cDNA was synthesized using the Illumina TruSeq

286 Stranded mRNA Sample preparation kit and dual indexing was used to multiplex sequencing of
287 samples. Library quality was assessed on the Agilent Tapestation D1000 DNA High Sensitivity
288 assay and quantified using the Qubit 3.0 High Sensitivity dsDNA assay to ensure equimolar
289 pooling. A total of 42 libraries were sequenced across two (75bp PE) Illumina NextSeq500 High
290 Output sequencing runs.

291 Quality control was applied to paired-end libraries via Trimmomatic (v.0.39) (minimum
292 quality score 20 and minimum length 45bp) (Bolger et al. 2014). The trimmed reads were
293 aligned to the reference genome, *Geospiza fortis* (GeoFor_1.0, INSDC Assembly
294 GCA_000277835.1, Jul 2012), utilizing HISAT2 (v.2.2.1) (Kim et al. 2015). Read counts were
295 extracted from each alignment file via HTSeq (v.0.13.5) with the published reference annotation
296 (Anders et al. 2015).

297 Read counts and corresponding treatment/location assignments were imported into
298 RStudio (Bioconductor), and DESeq2 (v.1.32.0) was used to identify differentially expressed
299 genes among nestling groups (Soneson et al. 2015; Love et al. 2014). The factorial design with
300 an interaction term (~site + treatment + site:treatment) compared urban and non-urban sites, and
301 sham-fumigated and fumigated treatments (Fig. S2). *P*-values derived from the Wald test were
302 corrected for multiple testing using the Benjamini and Hochberg method (Love et al. 2014). We
303 selected genes with an adjusted *P*-value less than 0.1. Initial functional annotations were
304 imported via biomaRt (v.2.48.3) with Ensembl (bTaeGut1_v1.p) (Durinck et al. 2009). An
305 enrichment and depletion (two-sided hypergeometric test) *immune system process* Gene
306 Ontology (GO) enrichment analysis was performed on differentially expressed genes of sham-
307 fumigated urban and non-urban nestlings to predict potential immune function pathways.
308 *Taeniopygia guttata* (zebra finch) GO annotations were sourced from UniProt GOA and
309 referenced within ClueGO (v.2.5.9) and CluePedia (v.1.5.9), plug-ins for Cytoscape (v.3.8.2)

310 (Bindea et al. 2009; Bindea et al. 2013; Shannon et al. 2003). Terms with a *P*-value < 0.1 were
311 considered significantly enriched.
312 A co-expression analysis was conducted via WGCNA (v.1.71) in RStudio (Langfelder
313 and Horvath 2008). Binary and quantitative trait information was imported along with
314 normalized RNA-Seq counts. A soft-thresholding power of 6 was chosen based on the criterion
315 of approximate scale-free topology and sample size, and eigengene significance was calculated
316 for each module. Within Cytoscape, a ClueGO network (referencing zebra finch) was
317 constructed to visualize key drivers in the context of immune response.
318
319 *Statistical analyses*
320 Statistical analyses of field data (i.e., non-gene expression data) were conducted in R
321 (2021, v.1.4.1103) and figures were created in Prism (2021, v.9.2.0). For dependent variables
322 with continuous data, we used a Shapiro-Wilk test for normality then chose the appropriate
323 distribution for the analysis. Since mass and tarsus were highly correlated ($R^2 = 0.74$, $P <$
324 0.0001), we calculated the scaled mass index, which is a standardized metric of body condition
325 (Peig and Green 2009). Bill surface area was also calculated from bill length, width, and depth
326 using a modified equation for the surface area of a cone (Greenberg et al. 2012).
327 Linear mixed effects models (LMMs) with nest as a random effect were used to analyze
328 the effect of location on parasite volume, effect of parasite intensity on parasite size, and effect
329 of Julian hatch day on parasite size. LMMs were used to determine the effect of site, treatment,
330 and their interaction on scaled mass index, bill size, hemoglobin levels, and glucose levels, with
331 nest as a random effect. Age and Julian hatch day were included as covariates when they
332 contributed significantly ($P < 0.05$) to the model (age only: bill size; Julian hatch day only:
333 glucose; neither: scaled mass index, hemoglobin levels). LMMs were also used to determine the

334 effect of site and sample type (nestling feces vs. fly) on $\delta^{15}N$, $\delta^{14}C$, and C:N, with sample
335 replicate as a random effect.

336 Generalized linear models (GLMs) were used to analyze the effect of location on parasite
337 abundance (Poisson distribution), the effect of location on Julian hatch day (Gamma
338 distribution), the effect of location and Julian hatch day on brood size (Gamma distribution), and
339 effect of Julian hatch day on parasite abundance (Poisson). GLMs were also used to determine
340 the effect of site, treatment, and their interaction on fledging age for the nest (log transformed;
341 Gaussian). A GLM with binomial errors for proportional data (i.e., a logistic regression) was
342 used to determine the effect of site, treatment, and their interaction on fledging success with
343 Julian hatch day as a covariate.

344 Analyses were conducted with the lmer function (LMMs) and glm function (GLMs)
345 using the lme4 package in R (Bates et al. 2015). Probability values were calculated using log-
346 likelihood ratio tests using the Anova function in the car package (Fox and Weisberg 2019). For
347 one-way and two-way ANOVAs, we used type II and type III sum of squares, respectively.
348

349 **Results**

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

351 Urban and non-urban finches initiated breeding in early February 2019. The first urban
352 nests hatched approximately seven days before the first non-urban nest. Although Julian hatch
353 day was, on average, earlier for urban nests (mean ± SE: 86.24 ± 3.99 days) compared to non-
354 urban nests (94.17 ± 4.07 days), Julian hatch day did not differ significantly between sites (χ^2 =
355 1.85, P = 0.17). Furthermore, site, Julian hatch day, and their interaction did not significantly
356 affect brood size (urban: 2.66 ± 0.18 nestlings, n = 38 nests; non-urban: 2.87 ± 0.17, n = 30
357 nests; site: χ^2 = 0.44, P = 0.51; hatch day: χ^2 = 0.68, P = 0.41; interaction: χ^2 = 1.12, P = 0.29).

358 Eight nests were classified as depredated, either due to direct evidence of depredation (e.g., body
359 parts found) or because they were likely depredated (e.g., all nestlings disappeared over a short
360 period of time and prior to the typical fledging window; Table 1).

361 The experimental treatment of nests with permethrin was nearly 100% effective at
362 removing flies (Fig. 1A; Table 1); only one permethrin-treated urban nest had eight flies after the
363 treatment. Water-treated nests were naturally parasitized by 27.00 (± 5.20) flies in the non-urban
364 area and 15.95 (± 3.15) flies in the urban area (Fig. 1A; Table 1). One water-treated nest
365 disappeared due to predation after the nestlings were banded and therefore we could not quantify
366 parasitism. Fourteen of 17 (82.35%) non-urban nests and 17 of 21 (80.95%) urban nests
367 contained at least one parasite. Within the sham-fumigated treatment, non-urban nests had
368 significantly fewer flies than urban nests ($\chi^2 = 54.49$, $P < 0.0001$). Julian hatch day did not
369 significantly predict nest parasite abundance ($\chi^2 = 1.58$, $P = 0.21$).

370 Parasite volume was larger in urban nests compared to non-urban nests (Fig. 1B; $\chi^2 =$
371 5.85, $P = 0.02$; Table 1). Urban fly pupae were, on average, 26% larger than non-urban fly
372 pupae(urban: 100.30 ± 7.62 mm^3, non-urban: 81.61 ± 9.29 mm^3). This difference was not related
373 to competition among flies within the nest because parasite intensity was not related to parasite
374 size ($\chi^2 = 0.01$, $P = 0.97$). Rather, the nest hatch day predicted parasite size, with earlier nests
375 having larger flies compared to later nests (Fig. 1C; $\chi^2 = 11.01$, $P < 0.001$).

376

377 *Effect of location and landmarks on nesting and parasite abundance*

378 Of all urban nests (sham-fumigated and fumigated), one nesting hotspot was identified
379 within the urban area ($n = 37$ total nests; $n = 19$ hotspot nests), which was at the airport (Fig. S1).
380 The hotspot grid resulted in 14 cells (78 m x 78 m) with 90% confidence of being a hotspot, with
381 *Z*-scores ranging between 2.90 and 6.02. The remaining cells in the study area were not

382 identified as significant nesting hot- or cool-spots. Of the 19 sham-fumigated nests within the
383 nesting hotspot, seven contained flies, and all but one of these was located near a bench.
384 Hotspots were not identified for fledging success, nor did we identify parasite hotspots among
385 sham-fumigated nests.
386
387 *Effect of parasitism and urbanization on nestling health*
388 Nestlings from sham-fumigated nests had, on average, lower body condition (i.e., scaled
389 mass index) than nestlings from fumigated nests (Table S1; $\chi^2 = 4.01$, $P = 0.045$). Site and the
390 interaction between site and treatment did not significantly affect body condition (site: $\chi^2 = 0.36$,
391 $P = 0.55$; interaction: $\chi^2 = 2.20$, $P = 0.14$). Treatment and site did not significantly affect bill
392 surface area (site: $\chi^2 = 0.13$, $P = 0.72$; treatment: $\chi^2 = 0.71$, $P = 0.40$). The interaction between
393 treatment and site had a marginally non-significant effect on bill surface area ($\chi^2 = 3.09$, $P =$
394 0.08); fumigated nestlings from non-urban areas had larger bill surface area than nestlings from
395 urban areas but the bill surface area for sham-fumigated nestlings from urban and non-urban
396 nests did not significantly differ. Most nestlings from non-urban, sham-fumigated nests died
397 before they could be measured; only 13 (of 44) non-urban nestlings from six sham-fumigated
398 nests survived to be measured.
399 Site alone did not significantly affect nestling hemoglobin levels (Fig. 2; Table S1; $\chi^2 =$
400 1.06, $P = 0.30$). Overall, sham-fumigated nestlings had lower hemoglobin levels than fumigated
401 nestlings ($\chi^2 = 18.82$, $P < 0.0001$). Furthermore, the interaction between treatment and site
402 affected hemoglobin levels ($\chi^2 = 6.16$, $P = 0.01$), with nestlings from sham-fumigated nests in
403 the non-urban area having, on average, 32% lower levels than nestlings from the other treatments
404 (i.e., sham-fumigated urban, fumigated urban, and fumigated non-urban nestlings).

405 Overall, nestlings from fumigated nests had higher glucose levels than nestlings from
406 sham-fumigated nests (Table S1; $\chi^2 = 9.75$, $P = 0.002$) and non-urban nestlings had higher
407 glucose levels than urban nestlings ($\chi^2 = 6.87$, $P = 0.009$). Additionally, the interaction between
408 treatment and site had an effect on nestling glucose levels ($\chi^2 = 9.34$, $P = 0.002$), with urban
409 nestlings from fumigated and sham-fumigated nests maintaining similar levels but non-urban
410 nestlings from fumigated nests having higher levels than sham-fumigated nestlings. Nestlings
411 from sham-fumigated nests in the non-urban area had, on average, 26% lower glucose levels than
412 nestlings from the other treatments (i.e., sham-fumigated urban, fumigated urban, and fumigated
413 non-urban nestlings).

414 Fledging success of urban and non-urban nestlings did not differ significantly ($\chi^2 = 0.67$,
415 $P = 0.41$), but overall, parasitism reduced fledging success (Fig. 1A; Table S1; $\chi^2 = 44.30$, $P <$
416 0.0001). The interaction between treatment and site affected fledging success ($\chi^2 = 8.28$, $P =$
417 0.004); survival of fumigated nestlings did not differ between sites but survival of sham-
418 fumigated nestlings was lower in non-urban areas, compared to urban areas (Table 1). Age at
419 fledging did not differ significantly between treatments ($\chi^2 = 0.20$, $P = 0.66$) but differed
420 between locations ($\chi^2 = 3.71$, $P = 0.05$); urban nestlings left the nest approximately one day later
421 than non-urban nestlings. The interaction between treatment and site did not significantly affect
422 fledging age ($\chi^2 = 0.10$, $P = 0.75$).

423

424 *Effect of urbanization on finch and parasite diet*

425 Flies were enriched in $\delta^{15}N$ compared to nestling feces ($\chi^2 = 164.94$, $P < 0.0001$) and all
426 urban samples were enriched in $\delta^{15}N$ compared to non-urban samples ($\chi^2 = 108.13$, $P < 0.0001$;
427 Fig. 3A). However, the interaction between sample type and site did not significantly affect $\delta^{15}N$
428 ($\chi^2 = 0.00$, $P = 0.99$). Nest parasite abundance correlated negatively with $\delta^{15}N$ values ($\chi^2 = 5.69$,

429 $P = 0.02$; Fig. 3B). Nestling feces were enriched in $\delta^{13}C$ compared to flies ($\chi^2 = 11.21$, $P =$
430 0.0008). However, site or the interaction between sample type and site did not affect $\delta^{13}C$ (site:
431 $\chi^2 = 0.43$, $P = 0.51$; interaction: $\chi^2 = 1.16$, $P = 0.28$). Nestling feces had a higher carbon to
432 nitrogen ratio (C:N), compared to flies ($\chi^2 = 21.60$, $P < 0.0001$). However, site and the
433 interaction between sample type and site did not affect C:N (site: $\chi^2 = 2.04$, $P = 0.15$; interaction:
434 $\chi^2 = 0.74$, $P = 0.39$).
435
436 *Sequencing, Quality Control, and Alignment*
437 In total, 42 paired-end nestling libraries were constructed, including 11 urban sham-
438 fumigated, five non-urban sham-fumigated , 14 urban fumigated, and 12 non-urban sham-
439 fumigated nestlings (1 nestling per nest). Altogether, the paired-reads post-QC ranged between
440 15.86M and 42.16M and alignment rates against the *Geospiza fortis* genome ranged from
441 80.74% to 91.81% (Table S2).
442
443 *Differentially Expressed Genes*
444 A pairwise differential expression analysis observing two sites (urban and non-urban) and
445 two treatments (fumigated and sham-fumigated), with an interaction, was conducted (File S1). A
446 total of 5,123 genes ($P\text{-}adj < 0.1$) were differentially expressed in sham-fumigated finches across
447 the urban (up-regulated) and non-urban (down-regulated) sites, of which, 2,521 were up-
448 regulated and 2,602 were down-regulated. Altogether, 57 genes demonstrated strong expression
449 patterns (± 4 fold change), including frizzled class receptor 10 FZD10 (+16.20 fold change), a
450 primary receptor for Wnt signaling, leucine rich repeat and Ig domain containing 3 LINGO3
451 (+5.35 fold change), which has been shown to regulate mucosal tissue regeneration in humans
452 and promote wound healing, adenosine deaminase ADA (-8.17 fold change), associated with

453 hemolytic anemia, and hepatic leukemia factor HLF (-5.64 fold change), known to influence the
454 renewal of hematopoietic stem cells (Wang et al. 2016; Zullo et al. 2021; Chen & Mitchell
455 1994; Komorowska et al. 2017). In fumigated finches, only two genes were up-regulated in the
456 urban site, bisphosphoglycerate mutase BPGM (+3.24 fold change), a regulator of erythrocyte
457 metabolism and hemoglobin in red blood cells, and zinc finger protein GLIS1 (+3.40 fold
458 change), which has been significantly associated with bill length (Xu et al. 2020; Lundregan et
459 al. 2018). There were no significantly down-regulated genes.

460 When comparing sham-fumigated versus fumigated finches in the urban site, a total of
461 768 genes were differentially expressed, with 570 being up-regulated in sham-fumigated birds,
462 and 198 down-regulated. The vast majority of genes showed moderate-to-low fold-change
463 patterns, with only three exhibiting strong expression patterns: inorganic pyrophosphate transport
464 regulator ANKH (+4.43 fold change), prokineticin 2 PROK2 (+4.38 fold change) and plexin A2
465 PLXNA2 (-4.08 fold change).

466 By comparison, sham-fumigated versus fumigated finches in the non-urban site yielded
467 7,064 differentially expressed genes, 3,709 of which were up-regulated in sham-fumigated birds,
468 and 3,355 down-regulated. Again, the majority of genes exhibited moderate-to-low fold-change,
469 with only 314 displaying strong expression patterns. This included an assembly factor for spindle
470 microtubules ASPM (+6.34 fold change), which has an apparent role in neurogenesis and
471 neuronal development (Nam et al. 2010) (Fig. S3-S4).

472

473 *Gene enrichment related to host defense mechanisms*

474 Gene enrichment of sham-fumigated nestlings from the urban and non-urban site was
475 compared to identify potential host defense mechanisms to explain the expression patterns
476 observed. Analysis of enriched *immune system process* Gene Ontology (GO) terms revealed 21

477 significant ($P < 0.05$) up-regulated terms (urban) and 12 significant down-regulated terms (non-
478 urban) categorized by resistance (adaptive and innate) and tolerance (File S2).

479 First, we examined enrichment patterns related to *adaptive immunological resistance*
480 (Fig. 4A). The urban sham-fumigated nestlings exhibited strong enrichment of lymphocyte
481 differentiation ($P < 0.02$) and T cell pathways related to regulation of CD4-positive, alpha-beta T
482 cell activation ($P < 0.01$) and NK T cell differentiation ($P < 0.05$). By comparison, non-urban
483 sham-fumigated nestlings yielded 21 significant adaptive immunity terms. Of those, ten were
484 related to Ig antibodies, including positive regulation of isotype switching to IgG isotypes ($P <$
485 0.001) and somatic hypermutation of immunoglobulin genes ($P < 0.001$). The rest related to B
486 cell proliferation ($P < 0.01$) and activation ($P < 0.05$), leukocyte differentiation ($P < 0.04$) and
487 proliferation ($P < 0.05$), regulation of antigen receptor-mediated signaling pathways ($P < 0.04$)
488 and germinal center formation ($P < 0.01$).

489 We next examined enrichment patterns related to *innate immunological resistance* (Fig.
490 4B). Urban sham-fumigated nestlings were enriched for both type I interferon mediated signaling
491 ($P < 0.03$) and toll-like receptor 9 signaling ($P < 0.05$). Among the non-urban nestlings, only
492 negative regulation of innate immune response ($P < 0.07$) was enriched.

493 Finally, we examined enrichment related to *tolerance* (Fig. 4C). Urban sham-fumigated
494 nestlings had three significantly enriched terms specific to red blood cells, including erythrocyte
495 differentiation ($P < 0.001$) and development ($P < 0.001$), as well as enucleate erythrocyte
496 differentiation ($P < 0.01$). Among non-urban nestlings, regulation of megakaryocyte
497 differentiation yielded a *P*-value less than 0.07.

498

499

500

501 *Co-expression Analysis*

502 A differential co-expression analysis was conducted to provide novel insights on other
503 traits that may explain gene expression patterns in nestling finches. Correlations in transcript
504 levels across 12 traits (urban, sham-fumigated, hemoglobin level, glucose level, alive, $\delta^{13}C$,
505 $\delta^{15}N$, C:N, days old, number of flies in nest, bill surface area and scaled mass) introduced 44
506 modules (File S3; Fig. S5-S8). Nestling survival ("alive" trait) presented the most significant
507 correlations across the gene set, as illustrated by module A ($r = 0.67$, $P < 0.0001$) and module B
508 ($r = -0.82$, $P < 0.0001$) respectively (Fig. 5). From the 2,338 total genes included in module A,
509 43 yielded 19 significantly enriched *immune system process* terms related to T-cell activation,
510 lymphocyte differentiation, and negative regulation of hemopoiesis. Comparatively, 55 of the
511 3,422 genes in module B activated 21 immune pathways related to somatic hypermutation of
512 immunoglobulin genes, leukocyte mediated cytotoxicity and interestingly, erythrocyte
513 differentiation.

514 Apart from mortality, other traits were strongly correlated with module A and B.
515 Specifically, module A was negatively correlated with parasitism ($r = -0.59$, $P < 0.0001$) and
516 nest parasite abundance ($r = -0.74$, $P < 0.0001$), and positively correlated with hemoglobin level
517 ($r = 0.66$, $P < 0.0001$), glucose level ($r = 0.48$, $P < 0.001$) and $\delta^{13}C$ ($r = 0.32$, $P < 0.04$).
518 Specifically, module B was positively correlated with parasitism ($r = 0.57$, $P < 0.0001$) and nest
519 parasite abundance ($r = 0.62$, $P < 0.0001$), and negatively correlated with hemoglobin level ($r = -$
520 0.52, $P < 0.0004$), glucose level ($r = -0.55$, $P < 0.0002$), $\delta^{13}C$ ($r = -0.34$, $P < 0.03$) and scaled
521 mass ($r = -0.45$, $P < 0.003$). By comparison, although there were no notable correlations to
522 urbanization in the described modules, the enriched immune pathways better explain patterns of
523 expression observed in urban and non-urban sham-fumigated finches.

524

525 **Discussion**

526 Our study found that urban living partially rescues nestling small ground finches from the
527 lethal effects of parasitism by invasive avian vampire flies. Survival of sham-fumigated nestlings
528 did not differ significantly between areas, which suggests that urbanization alone does not affect
529 fledging success. Urban nests had significantly fewer flies than non-urban nests, such that even
530 sham-fumigated urban nestlings survived. These results indicate that urban nestlings have
531 effective resistance and tolerance mechanisms to deal with avian vampire flies. Differences in
532 diet (measured using stable isotopes) between locations could explain why urban nestlings see
533 fewer negative fitness consequences, but these results are correlative. Gene expression analyses
534 revealed that resistance and tolerance mechanisms might underlie differences in parasite effects.
535 Parasitized urban nestlings differentially expressed genes within pathways associated with
536 tolerance and innate immunological resistance, compared to sham-fumigated non-urban
537 nestlings. In contrast, sham-fumigated non-urban nestlings differentially expressed genes within
538 pathways associated with adaptive immunological resistance. The gene expression results
539 suggest that non-urban nestlings are investing in adaptive immunity, but that this type of
540 response is not an effective defense mechanism against the parasite since few nestlings survived.

541 Overall, our field data suggest that the urban finch population is investing in resistance
542 because urban nests had 40% fewer flies and higher nestling survival than non-urban nests. Gene
543 expression profiles showed that urban nestlings differentially express innate immune genes
544 associated with pro-inflammatory cytokines, specifically type-1 interferons (IFNs), compared to
545 non-urban nestlings. When larval flies chew through the skin of their hosts, effective
546 inflammation by the host (thickening the skin and restricting blood flow) can prevent
547 ectoparasite feeding (reviewed in Owens et al. 2010). Although other avian urbanization studies
548 have not observed changes in IFNs, they have found other enhanced innate immune responses

549 within urban populations. For example, Watson et al. (2017) observed overrepresented genes
550 involved in the secretion and receptor-binding of cytokines in urban great tits (*Parus major*)
551 compared to non-urban tits. Additionally, urban nestling black sparrowhawks (*Accipiter*
552 *melanoleucus*) had a stronger innate response to an immune challenge compared to non-urban
553 nestlings (Nwaogu et al. 2023). These studies provide evidence that urban living could confer an
554 advantage against parasitism. Our study further supports this idea, as shown by the heightened
555 innate immune response in urban nestlings, and also links this response with a decrease in
556 parasite abundance.

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

569 Although sham-fumigated non-urban nestlings did not exhibit significantly enriched
570 innate immune pathways, they did differentially express genes involved in pathways related to
571 the adaptive immune response. Specifically, T-cell, B-cell, and Ig pathways were expressed, but
572 without successfully conferring resistance since almost all of the nestlings died. This

upregulation could be the final effort by the finches' immune system to deal with the parasite before it becomes physiologically costly. In contrast, urban finch nestlings expressed more 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 may be able to resist parasites (Cornet, Bichet, Larcombe, Faivre, & Sorci, 2014; Howick & Lazzaro, 2014; Lochmiller & Deerenberg, 2000; Sheldon & Verhulst, 1996; Sternberg et al., 2012; Svensson, Råberg, Koch, & Hasselquist, 1998). 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 of 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 protein, which could include food items such as chicken and fish, found at outdoor restaurants in town. Higher $\delta^{15}N$ values also correlated with lower parasite intensities, which supports the idea that diet might influence host defenses. For example, meat has higher protein concentrations than plants and supplemented protein can increase the concentration of cellular immune cells (e.g., eosinophils, globule leukocytes and mast cells) (reviewed in Coop and Kyriazakis, 2001). Although our results suggest that urban finches are more physiologically resistant to the flies than non-urban

finches, another potential explanation is that nest material affects the survival of flies. For example, urban Darwin's finches incorporate cigarette butts into their nests (Harvey et al. 2021), which can affect parasite abundance in other systems (Suarez-Rodriguez et al. 2013). Future studies could explore the idea that nest material could contribute to increased resistance in urban finches.

Some urban nests had 100% nestling survival despite high parasite abundances (up to 48 flies), indicating that these urban nestlings are tolerant of flies. Studies have demonstrated that low finch survival in response to vampire flies is likely related to exsanguination (i.e., high blood loss) (Fessl et al. 2006). One explanation for increased tolerance is that urban nestlings have effective blood recovery when sham-fumigated and thus tolerate parasitism. This hypothesis is corroborated with our results that urban finches have more hemoglobin (oxygenated blood) when sham-fumigated compared to non-urban birds. Gene expression profiles suggest that urban nestlings differentially expressed genes within pathways associated with red blood cell production compared to non-urban nestlings when sham-fumigated. Furthermore, nestlings that survived had higher gene expression of blood cell production than nestlings that died, which was observed across urban and non-urban sites. Galápagos mockingbirds are also relatively tolerant of avian vampire flies (Knutie et al. 2016), but this tolerance is lost during dry years with low food availability (McNew et al. 2019). The working hypothesis is that mockingbirds are a larger-bodied species and that larger hosts might be more tolerant of avian vampire flies (McNew and Clayton 2018). However, our study suggests that even smaller-bodied hosts can tolerate avian vampire flies, and there is likely an alternative explanation. The hormone erythropoietin is produced primarily by the kidneys and works together with iron to induce erythropoiesis, which is the production of red blood cells. One explanation for the lack of tolerance in non-urban nestlings is that these nestlings are not receiving sufficient amounts of iron or are not producing

621 enough erythropoietin. Urban nestlings have higher $\delta^{15}N$ values, which is likely because they are
622 feeding on more iron-rich meat products than non-urban birds. Thus, this difference in diet could
623 explain the increased tolerance related to red blood cell recovery, but requires further studies on
624 iron and nestling endocrinology. Finally, although non-urban nestlings did not exhibit any
625 significantly enriched pathways related to tolerance, we found that adenosine deaminase (ADA)
626 was significantly up-regulated. Interestingly, overexpression of ADA in red blood cells has been
627 shown to cause hemolytic anemia (Chen & Mitchell 1994), which could be additionally
628 responsible for non-urban finch mortality.

629 Although survival of urban nestlings in response to flies is higher than non-urban
630 nestlings, some urban nests still failed. Therefore, not all urban finches have effective defenses
631 against avian vampire flies. Our urban field location is environmentally heterogeneous with nests
632 found in areas of high tourist activity, residential areas, a naval base, and an airport. Our spatial
633 analysis did not find any distinct patterns for parasite abundance or fledging success related to
634 these landmarks. However, human food type, abundance, and reliability vary across these
635 locations and over time. Future research should consider how the heterogeneity in urban areas
636 may favor more phenotypic plasticity in effective defenses and/or hinder adaptations in the
637 population.

638 Heterogeneity across different urban areas could also impact finch-fly interactions on
639 other islands in the Galapagos. Due to logistical limitations, replication of sites was not possible
640 across islands. However, site replication would be helpful to understand whether context
641 dependency exists across different urban areas. The Galapagos Islands have two other major
642 towns, including Puerto Ayora of Santa Cruz Island with approximately 12,000 human residents
643 and Puerto Villamil of Isabela Island with approximately 2,000 human residents (Memoria
644 Estadística Galápagos 2017). Human population size often scales with anthropogenic features

645 (e.g., artificial light at night, density of restaurants, number of vehicles) in urban areas, which
646 can proportionally affect bird species (reviewed in Isaksson 2018). Thus, replicating our study in
647 towns on other islands in the Galapagos could provide insight into whether the scale of
648 urbanization impacts finch-fly interactions differently.

649 Naïve hosts are thought to lack defenses against novel parasites, which can cause species
650 declines and extinctions (Daszak et al. 2000, Keesing et al. 2010, Atkinson and Lapointe 2009).
651 Over the past decade, studies have found that invasive avian vampire flies can cause up to 100%
652 mortality in Darwin's finch species across islands in the Galápagos (O'Connor et al. 2010, Koop
653 et al. 2010, Koop et al. 2013, O'Connor et al. 2014; Klendorfer and Dudaniec 2016; Knutie et al.
654 2016, McNew and Clayton 2018, Addesso et al. 2021). If humans cannot effectively control the
655 fly (e.g., Knutie et al. 2014) or host species do not evolve or develop defenses, some species may
656 even go extinct (Fessl et al. 2010, Koop et al. 2013b). However, our study provides experimental
657 evidence that an urban population of finches is relatively well-defended against avian vampire
658 flies. Indeed, additional studies of urban and non-urban populations across years are needed to
659 determine the magnitude of the urban effect, but preliminary data from other years (2022, 2023)
660 and populations (Puerto Chino, San Cristobal) suggests that this pattern is observed beyond our
661 study (SAK prelim. data). To be clear, we are not suggesting that the Galápagos Islands should
662 be urbanized to increase defense against flies. Instead, the goal of our study is to demonstrate
663 that some finches can defend themselves against the flies through various mechanisms, which
664 can provide insight into management strategies (Ohmer et al. 2021).

665

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Data Availability Statement: Supplemental files 1-3 and details of full analysis, including intermediate files and supporting scripts, are publicly available at: https://gitlab.com/PlantGenomicsLab/galapagos-finch-rna-seq. All raw data are available on FigShare (DOI: available upon acceptance) and sequences have been uploaded to GenBank (BioProject accession number: PRJNA930453).

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914

915 **Figure legends**

916 **Fig. 1.** A) The influence of urbanization on mean (± SE) nest parasite abundance (number of
917 flies per nest) and % fledging success. Fledging success did not differ for fumigated nests in the
918 urban and non-urban area. In sham-fumigated nests, urban nests had, on average, fewer flies and
919 higher fledging success compared to non-urban nests. Each point represents an individual nest
920 and darker points indicate higher sample sizes. Diamonds represent the fumigated treatment and
921 squares represent the sham-fumigated treatment. B) Pupal volume (mm^3) was larger in the urban
922 area compared to the non-urban area. Each point represents a mean (± SE) volume for a nest. C)
923 Parasite volume decreased throughout the nesting season in both locations. Means and standard
924 error bars are represented in panels A and B.

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

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

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

939 Lymphocyte, Pattern Recognition, Pro-Inflammatory and Other. C) Tolerance categories, shown
940 in orange, were split into two sub-categories: Erythrocyte and Hematopoietic. Log10 (*P*-value)
941 significance of unique and shared terms is depicted a color saturation gradient (** = *P*-value <
942 0.05), and number of genes supporting each ontology term is represented by circle size.
943 **Fig. 5.** *Immune system process* Gene Ontology enrichment network of co-expression module A
944 (top) and B (bottom). WGCNA trait correlation is depicted on a scale from 0 (white) to 1 (red),
945 and negative trait correlation significance from 0 (white) to -1 (blue). WGCNA *P*-values are
946 shown in parentheses. The co-expressed genes are represented with ClueGO in pathway form.
947 The significance of enriched Gene Ontology terms is represented by the gray gradient. The size
948 of the circle represents the number of genes associated with the enriched term.
949

950 **Table 1.** Sample sizes for the effect of location (non-urban or urban) and parasite treatment

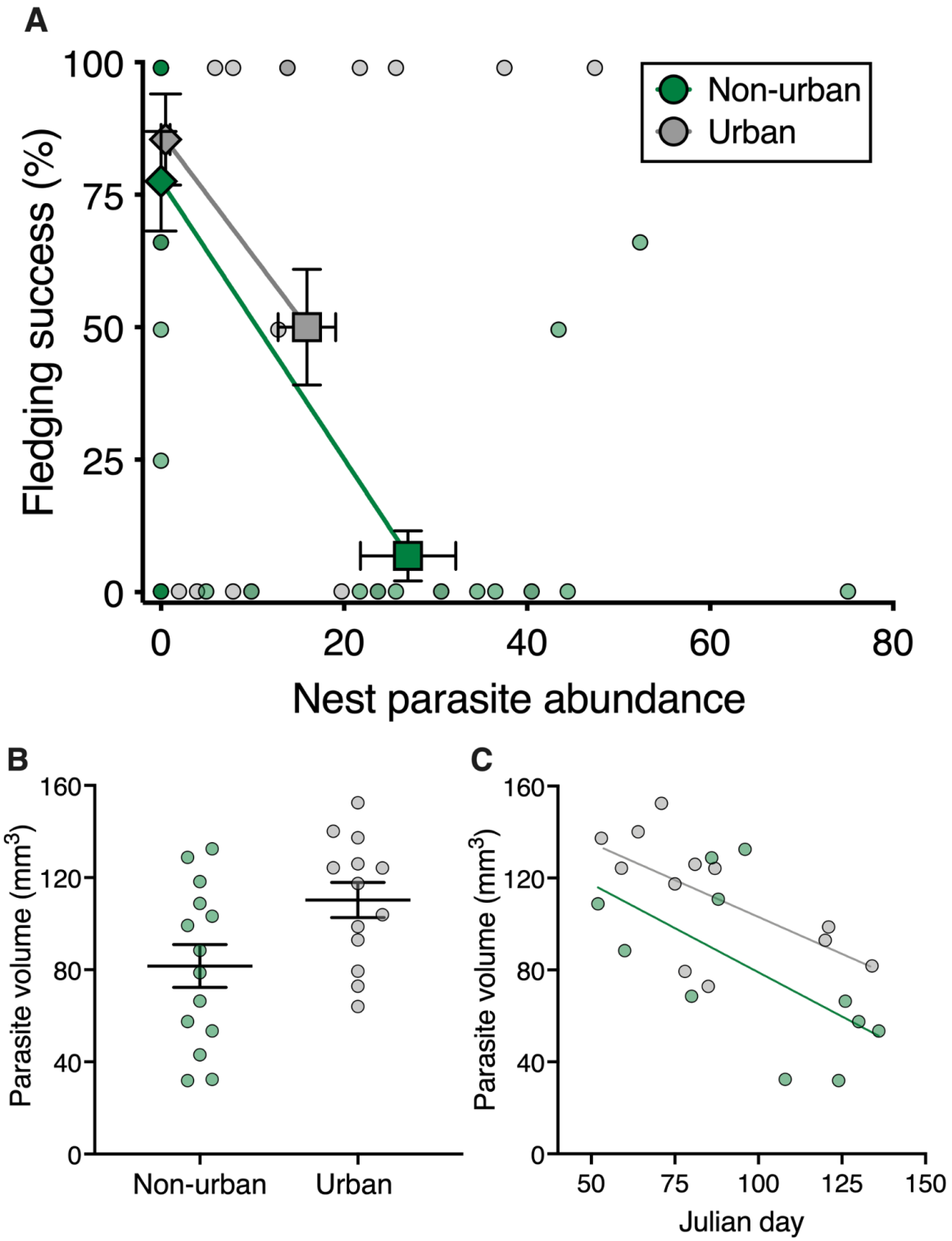
951 (fumigated or sham-fumigated) on nest, parasite, and nestling variables.

	Non-urban		**Urban**	
Sample sizes	**Fumigated**	**Sham-fumigated**	**Fumigated**	**Sham-fumigated**
# nests in experiment	13	17	16	22
# nests with fledging age	12	2	14	11
Individual fly sample sizes: # flies (# nests)				
Pupal size	NA	90 (14)	NA	93 (13)
Adult fly stable isotope values	NA	11 (11)	NA	7 (7)
Individual nestling sample sizes: # nestlings (# nests)				
Scaled mass index	27 (12)	13 (6)	37 (15)	31 (12)
Bill surface area	27 (12)	13 (6)	37 (15)	31 (12)
Hemoglobin levels	10 (10)	6 (6)	18 (14)	11 (9)
Glucose levels	16 (12)	6 (4)	18 (12)	15 (9)
Stable isotope values	11 (11)	2 (2)	12 (11)	9 (8)
Gene expression	14 (14)	5 (5)	12 (12)	11 (11)
Fledging success				
# nests depredated/total # of nests	1/13 (8%)	2/17 (12%)	2/16 (13%)	3/22 (14%)
# nests with at least one fledgling /total # of nests	12/13 (92%)	2/17 (12%)	14/16 (88%)	11/22 (50%)
# fledglings/total # of hatchlings	27/36 (75%)	4/50 (8%)	34/43 (79%)	29/58 (50%)

952

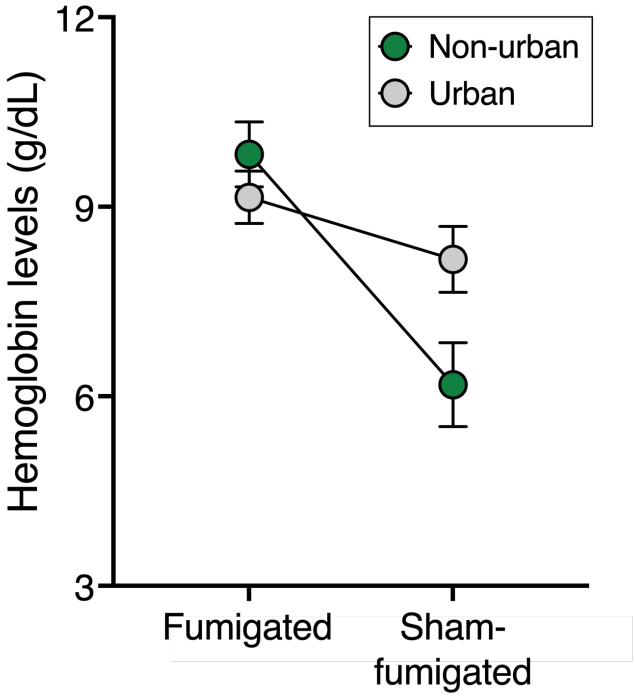
953

954 **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

955

956 **Fig. 2.**



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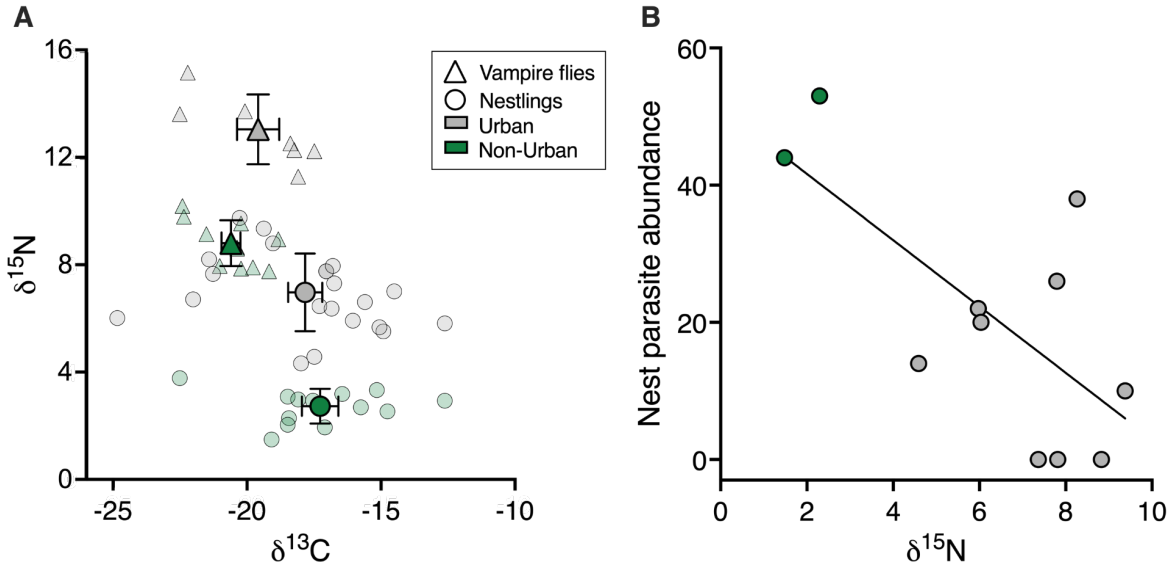
959 **Fig. 3.**


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

960

961 **Fig. 4.**

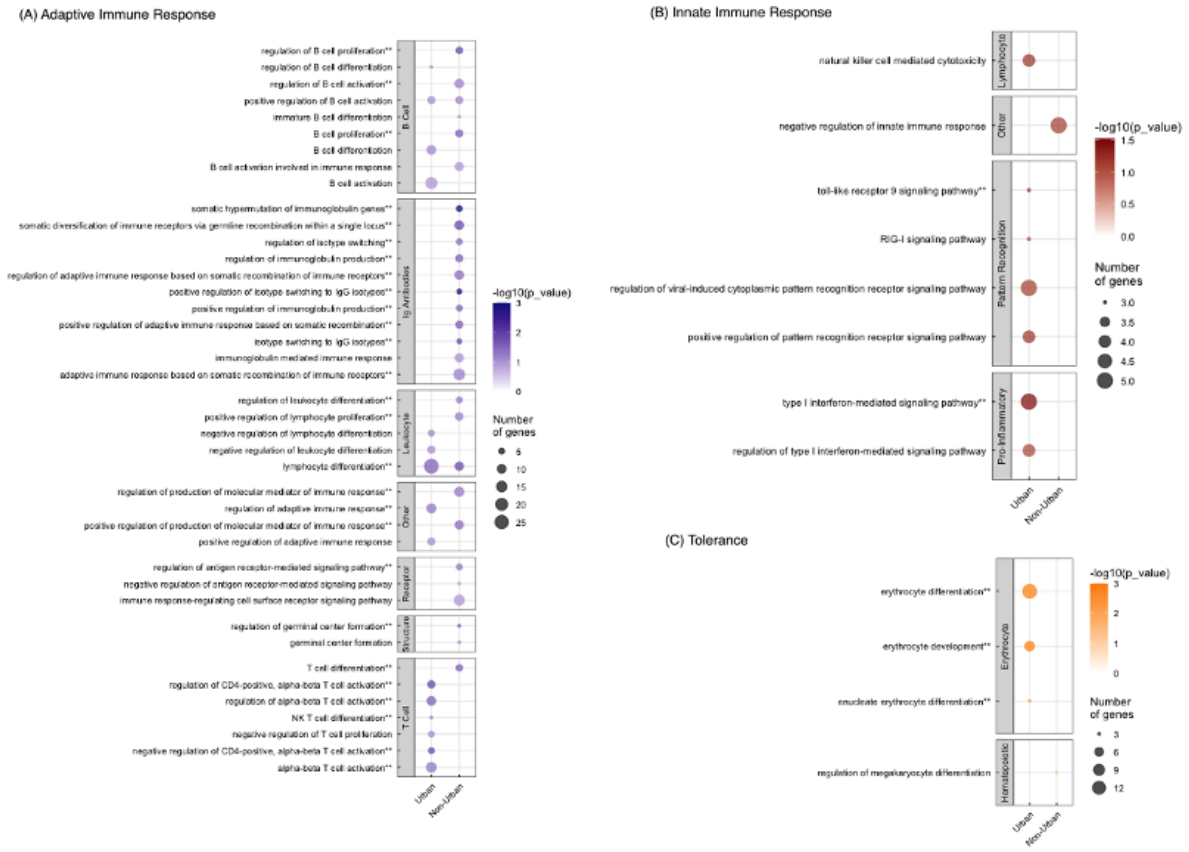


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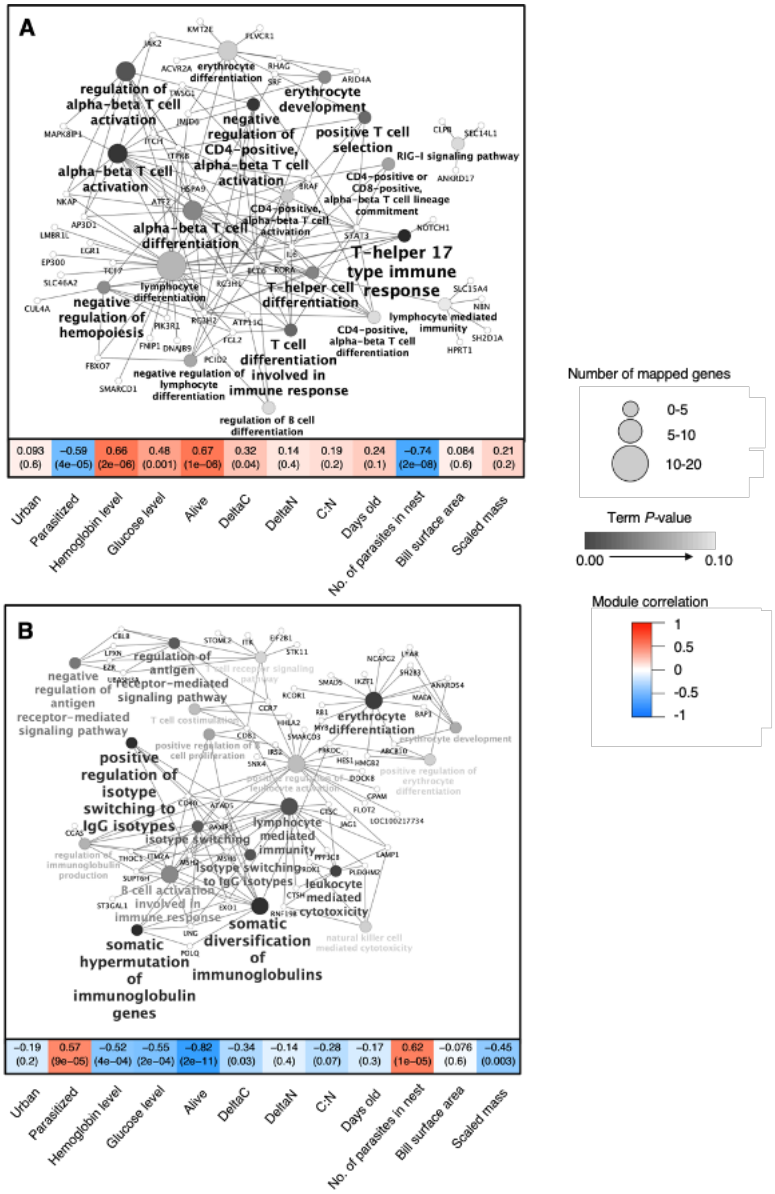
Fig. 5


A
regulation of alpha-beta T cell activation
erythrocyte differentiation
erythrocyte development
negative regulation of CD4-positive, alpha-beta T cell activation
positive T cell selection
RIG-I signaling pathway
alpha-beta T cell activation
CD4-positive or CD8-positive, alpha-beta T cell lineage commitment
CD4-positive, alpha-beta T cell activation
alpha-beta T cell differentiation
T-helper 17 type immune response
T-helper cell differentiation
lymphocyte differentiation
negative regulation of hemopoiesis
lymphocyte mediated immunity
CD4-positive, alpha-beta T cell differentiation
T cell differentiation involved in immune response
negative regulation of lymphocyte differentiation
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
Parasitized
Hemoglobin level
Glucose level
Alive
DeltaC
DeltaN
C:N
Days old
No. of parasites in nest
Bill surface area
Scaled mass
B
regulation of antigen receptor-mediated signaling pathway
negative regulation of antigen receptor-mediated signaling pathway
erythrocyte differentiation
positive regulation of isotype switching to IgG isotypes
lymphocyte mediated immunity
isotype switching
isotype switching to IgG isotypes
B-cell activation involved in immune response
leukocyte mediated cytotoxicity
somatic diversification of immunoglobulins
somatic hypermutation of immunoglobulin genes
−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
Parasitized
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