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RESEARCH ARTICLE

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 positive or negative synergistic 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 urban and non-urban locations and then characterized nestling health, fledging success, diet, and gene expression patterns related to host defense. Fledging success of non-parasitized nestlings from urban (79%) and non-urban (75%) nests did not differ significantly. However, parasitized, non-urban nestlings lost more blood, and fewer nestlings survived (8%) compared to urban nestlings (50%). Stable isotopic values (δ^{15}N) from urban nestling feces were higher than those from non-urban nestlings, suggesting that urban nestlings are consuming more protein. δ^{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.

KEYWORDS
Darwin's finch, ecoimmunology, gene expression, host defenses, invasive parasites, resistance, stable isotopes, tolerance, transcriptomics

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1 | 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 (Frick et al., 2010; Van Riper et al., 1986). However, not all hosts are susceptible to introduced parasites. The fitness of some hosts 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 et al., 2012; Miller et al., 2006; Råberg et al., 2007; Read et al., 2008). For example, parents from ectoparasite-infested nests reduce the cost of parasitism by feeding their offspring more than parents from non-parasitized nests (Christe et al., 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 exclusively, 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 against invasive parasites are highly variable among individuals and populations and often depend on both genetic variation and phenotypic plasticity (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 (Bosse et al., 2017; Murray et al., 2016; Start et al., 2018). Due to the high energetic cost of defense mechanisms, only hosts with sufficient food resources, such as in these urban locations, may be able to invest in defenses (Cornet et al., 2014; Howick & Lazzaro, 2014; Knutie, 2020; Lochmiller & Deerenberg, 2000; Sheldon & Verhulst, 1996; Sternberg et al., 2012; Svensson et al., 1998). Extra nutrients obtained from human-derived food, such as protein, can directly increase the production of immune cells (Coop & Kyriazakis, 2001; Strandin et al., 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 decline, and this effect might be especially apparent in the context of human-influenced environments (Atkinson & LaPointe, 2009; Van Riper et al., 1986). 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 they 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 & 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 & 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 (Cimadom et al., 2016; Koop, Le Bohec, et al., 2013; Koop, Owen, et al., 2013). This fly dramatically reduces the fledging success (i.e., ability to successfully leave the nest) of endemic Darwin's finches and, in some years, can result in 0% fledging success of parasitized nestlings (Koop, Le Bohec, et al., 2013; Koop, Owen, et al., 2013; O'Connor et al., 2014). However, some endemic Galápagos species, such as the Galápagos mockingbird (*Mimus parvulus*), are able to tolerate the effect of the fly because parents from parasitized nests feed their nestlings more than parents from non-parasitized nests to compensate for energy lost to the flies (Knutie et al., 2016). Because urban locations offer more consistent food, though likely of lower quality (De León et al., 2019), urban locations could amplify or dampen host defense strategies against this parasitic fly.

This study examined the effect of urbanization, avian vampire flies, and their interaction on nestling Darwin's finches. For our first objective, we investigated whether the effect of the flies on finch nestlings differed in urban and non-urban locations. We experimentally removed vampire flies from or allowed for parasitism in the nests of small ground finches (*Geospiza fuliginosa*; a species of Darwin's finch) in the urban (Puerto Baquerizo Moreno) and non-urban locations (Jardín de las Opuntias) on San Cristóbal Island, Galápagos. We then quantified nestling morphometrics, blood loss, glucose levels, and fledging success in response to parasitism across treatments and locations. 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 foods (De León et al., 2019), which lack many nutritional qualities required for an active immune system, urban nestlings might not be well-defended against the parasite. Similar to mockingbirds, increased food availability might also increase tolerance to parasitism in urban birds if we observe that

high parasite abundances do not negatively affect fledging success. Spatial variation of urban parasite abundance was also examined to determine whether parasite load had spatial structure within the urban location or was associated with various environmental features (e.g., restaurants).

Our second objective examined the molecular mechanisms that underlie the defense response to avian vampire flies in urban and non-urban nestlings. We characterized gene expression in the blood of ~8-day-old nestlings across parasite treatments and locations. Gene expression is the process in which gene information is turned into a functional product, which can affect phenotypes. For example, nestlings in urban locations may have more relative expression of genes in a pathway that affects erythrocyte production, which could account for the higher tolerance of flies. Co-expression profiles were also used to compare gene expression with the organismal traits of nestlings. If urban nestlings are better defended against parasites, we expect to observe increased expression of genes related to innate and adaptive resistance and/or tolerance of blood loss from parasites compared to non-urban nestlings. Consequently, urban, parasitized nestlings are expected to have better health and fledging success 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.

2 | METHODS

2.1 | Study system

We conducted our study between February 2019 and May 2019 in urban and non-urban locations in the dry lowland climatic zone of San Cristóbal (557 km^2) in the Galápagos Islands. The urban location encompasses the only city on San Cristóbal Island, Puerto Baquerizo Moreno (hereon, urban location) (−0.9067715°, −89.6061678°). This capital city is the second largest city in the Galápagos archipelago with a human population of 7199 (INEC, 2016) and measures 0.79 km^2 (~1.2 km by 0.62 km; Figure S1), which includes tourist and residential areas (Harvey et al., 2021). The urban location 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 introduced and established by humans.

The non-urban location is in Jardín de las Opuntias (hereon, non-urban location), which is a protected Galápagos National Park site located eight kilometers southeast of the urban location (−0.9491651°, −89.5528454°). This location measures 0.21 km^2 and covers 1.4 km of the main trail and 0.15 km to each side, does not contain any unnatural, human-built impermeable surfaces, and includes native plants, such as matazárno, acacia, and cacti. The non-urban location 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 on San Cristóbal commonly nest in both the urban and non-urban locations, generally between February and 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 locations, finches frequently incorporate trash and human hair into the construction of their nests (Harvey et al., 2021; Theodosopoulos & Gotanda, 2018). Finches produce clutches between 1 and 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.

2.2 | Nest parasite manipulation

Both field locations 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 (Cimadom et al., 2016), the nests were assigned to a control (hereon, sham-fumigated) or experimental (hereon, fumigated) treatment after a full clutch of finch eggs was laid. The treatment was initially determined by a coin flip, and the following nests were assigned an alternating treatment for each nest. Three non-urban nests and five urban nests were not found until after nestlings hatched (i.e., were <3 days old) and thus assigned to the control treatment and treated with water. 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 (Addesso et al., 2020; Fessl et al., 2006; Kleindorfer & Dudaniec, 2016; Knutie et al., 2014, 2016; Koop, Le Bohec, et al., 2013; Koop, Owen, et al., 2013; McNew et al., 2019) 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 feathers). 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.

2.3 | 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 nearest 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 s). We then transferred the fecal sac into a sterile 2 mL tube and kept it 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.

For up to three nestlings per nest, we also collected a small blood sample (<20 μL) from the brachial vein using a 30-gauge sterile needle and a heparinized capillary tube. Since nestlings are being fed upon by a hematophagic ectoparasite, we wanted to collect the smallest blood sample possible from each nestling. Therefore, we could not quantify all physiological metrics for every nestling. Ten microliters were used to quantify hemoglobin levels (g/dL) with a HemoCue® HB +201 portable analyzer (HemoCue America, USA) ($n = 1$–2 nestlings per nest). Five microliters were used to quantify glucose levels (mg/dL) with a glucometer (OneTouch, USA) ($n = 1$–2 nestlings per nest). Twenty microliters of whole blood were preserved in 180 μL of RNAlater ($n = 1$ nestling per nest); this preserved blood was kept at 4°C in a portable insulin refrigerator until we returned from the field. The sample was then vortexed and stored at 4°C for 24 h, according to the manufacturer's protocol, before being placed in a −20°C freezer while in the Galápagos (up to 2 months). The samples were then transported on ice packs (20 h) to the University of Connecticut, where they were stored for 2 months at −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 2 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.

2.4 | Quantifying parasite abundance

Within 2 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 (first, second, and third instars), pupae, and pupal cases were identified and counted to determine the total parasite abundance for each nest. The length and width (0.01 mm) of up to 10 pupae per nest were haphazardly measured with digital calipers. These measurements were used to calculate pupal volume ($V = \pi \times [0.5 \times \text{width}]^2 \times \text{length}$). 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 10 adult flies were collected and preserved in 95% ethanol for stable isotope analysis (see below for methods).

2.5 | 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 differences in C4 versus 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 finches. Feces were dried in an oven at 60°C for 24 h. Fly samples were rinsed three times in a 2:1 (vol/vol) chloroform/methanol mixture to remove surface oils, then rinsed with sterile water, and dried at 60°C for 24 h. After drying, samples were ground to a fine powder using a mortar and pestle. Up to 1 mg of each dried, homogenized sample was weighed into a tin capsule (Costech Analytical Technologies, Inc., USA). Capsules were folded and placed into a 96-well plate, then sent to the University of New Mexico Center for Stable Isotope Ratio Analysis (SIRA). The samples were run as duplicates on a Thermo Delta V mass spectrometer connected with a Costech 4010 Elemental Analyzer.

2.6 | Effect of location and landmarks on parasite abundance and fledging success

To explore whether location or landmarks (i.e., food markets, bakeries, benches, and restaurants) are associated with parasite abundance and fledging success, we conducted an optimized hotspot analysis in ArcGIS 10.8.1 (ESRI, 2020). First, a minimum convex polygon (MCP) was created to serve as our study location for spatial analysis and included all landmarks within the urban location. Nesting hotspots were then determined based exclusively on the spatial location of nests (i.e., clusters of finch nests rather than any other features on the landscape), which resulted in a grid of cells

that were characterized as hotspots or not within the MCP-derived study area. To learn about the characteristics of these nests, the Near Tool was used to identify and calculate the distance to the nearest landmark for all urban nest locations that were within the nesting hotspots. Specifically, we were interested in all landmarks associated with food, and there were 190 records of 303 landmarks available. We then extracted the locations of nests within hotspot locations and reviewed the landmark types associated with these nests. Finally, we conducted hotspot analyses based on parasite load (i.e., is there a hotspot of nest flies?) and also based on fledging (i.e., is there a hotspot of fledging success?). The hotspot analysis uses the Getis Ord-Gi statistic (Getis & Ord, 1992), which determines spatial clusters with either high or low values for the statistic (Fischer & Getis, 2010), corresponding to hot or cool spots, respectively.

2.7 | RNA extractions, sequencing, and bioinformatics

Total RNA was extracted from 20 μL of peripheral whole blood using a modified Tri-Reagent (Ambion, Invitrogen, USA) and Direct-zol RNA Miniprep Plus Kit (Zymo Research, USA) protocol (Harvey & Knutie, 2023). The samples were incubated at room temperature for 2 min and then centrifuged for 1 min at 8000 *g* to lightly pellet the blood. Preservation fluid (RNAlater; Ambion, Invitrogen) was pipetted off, leaving no more than ~15 μL of preservative, and 500 μL of Tri-Reagent were added along with a sterile 5 mm stainless steel bead (Thomson, USA). The samples were then vortexed for 30 s before adding an additional 500 μL of Tri-Reagent. The sample was then vortexed for 10 min at room temperature. The phase separation portion of the Tri-Reagent protocol was then followed, and the upper aqueous phase (500 μL) was transferred to new microcentrifuge tubes. We then followed the manufacturer's protocol for the Direct-zol RNA Kit, beginning with the RNA purification step. We eluted total RNA using 50 μL of RNA/DNA-free water. We used a 4200 TapeStation and High Sensitivity RNA ScreenTape assays (Agilent, USA) to quantify total RNA concentration (ng/μL) and RNA integrity numbers (RIN^e) (Schroeder et al., 2006). RNA extracts were then stored at −80°C until sequencing. The mean RNA concentration was 74.62 ng/μL (range: 15.2–246.0) and the mean RIN^e was 9.33 (range: 7.8–10), with all samples above the minimum RIN^e cutoff (=7.0) for successful sequencing.

A poly-A tail-binding bead-based approach was used to reduce ribosomal RNA contamination. First- and second-strand cDNA were synthesized using the Illumina TruSeq Stranded mRNA Sample Preparation Kit, and dual indexing was used to multiplex the sequencing of samples. Library quality was assessed on the Agilent Tapestation D1000 DNA High Sensitivity assay and quantified using the Qubit 3.0 High Sensitivity dsDNA assay to ensure equimolar pooling. A total of 42 libraries were sequenced across two (75 bp PE) Illumina NextSeq500 High Output sequencing runs.

Quality control was applied to paired-end libraries via Trimmomatic (v.0.39) (minimum quality score 20 and minimum length 45 bp) (Bolger et al., 2014). The trimmed reads were aligned to the reference genome, *Geospiza fortis* (GeoFor_1.0, INSDC Assembly GCA_000277835.1, Jul 2012), utilizing HISAT2 (v.2.2.1) (Danecek et al., 2021; Kim et al., 2015). Read counts were extracted from each alignment file via HTSeq (v.0.13.5) with the published reference annotation (Anders et al., 2015).

Read counts and corresponding treatment and location assignments were imported into RStudio (Bioconductor), and DESeq2 (v.1.32.0) was used to identify differentially expressed genes among nestling groups (Love et al., 2014; Soneson et al., 2015). The factorial design with an interaction term (~location + treatment + location:treatment) compared urban and non-urban locations and sham-fumigated and fumigated treatments (Figure S2). *p*-values derived from the Wald test were corrected for multiple testing using the Benjamini and Hochberg methods (Love et al., 2014). We selected genes with an adjusted *p*-value less than 0.1. Initial functional annotations were imported via biomaRt (v.2.48.3) with Ensembl (bTaeGut1_v1.p) (Durinck et al., 2009). An enrichment and depletion (two-sided hypergeometric test) *immune system process* Gene Ontology (GO) enrichment analysis was performed on differentially expressed genes of sham-fumigated urban and non-urban nestlings to predict potential immune function pathways. *Taeniopygia guttata* (zebra finch) GO annotations were sourced from UniProt GOA and referenced within ClueGO (v.2.5.9) and CluePedia (v.1.5.9), plug-ins for Cytoscape (v.3.8.2) (Bindea et al., 2009, 2013; Shannon et al., 2003). Terms with a $p < 0.1$ were considered significantly enriched.

A co-expression analysis was conducted via WGCNA (v.1.71) in RStudio (Langfelder & Horvath, 2008). Binary and quantitative trait information was imported along with normalized RNA-Seq counts. A soft-thresholding power of six was chosen based on the criterion of approximate scale-free topology and sample size, and eigengene significance was calculated for each module. Within Cytoscape, a ClueGO network (referencing zebra finch) was constructed to visualize key drivers in the context of immune response.

2.8 | Statistical analyses

Statistical analyses of field data (i.e., non-gene expression data) were conducted in R (2021, v.1.4.1103), and figures were created in Prism (2021, v.9.2.0). Since mass and tarsus were highly correlated ($R^2 = .74$, $p < .0001$), we calculated the scaled mass index, which is a standardized metric of body condition (Peig & Green, 2009). Bill surface area was also calculated from bill length, width, and depth using a modified equation for the surface area of a cone (Greenberg et al., 2012).

Linear mixed effects models (LMMs) with nest as a random effect were used to analyze the effect of location on parasite volume, the effect of parasite intensity on parasite volume, and the effect of Julian hatch day on parasite volume. LMMs were used to determine the effect of location, treatment, and their interaction on scaled mass index, bill surface area, hemoglobin levels, and glucose levels, with nest as a random effect. Age and Julian hatch day were included as

covariates when they contributed significantly ($p < .05$) to the model (age only: bill surface area; Julian hatch day only: glucose; neither: scaled mass index, hemoglobin levels). LMMs were also used to determine the effect of location and sample type (nestling feces vs. fly) on $\delta^{15}N$, $\delta^{14}C$, and C:N, with sample replicate as a random effect; only one sample was tested per nest.

Generalized linear models (GLMs) were used to analyze the effect of location on parasite abundance (Poisson distribution), the effect of location on Julian hatch day (Gamma distribution), the effect of location and Julian hatch day on brood size (Gamma), and the effect of Julian hatch day on parasite abundance (Poisson). GLMs were also used to determine the effect of location, treatment, and their interaction on fledging age for the nest (Gaussian). A GLM with binomial errors for proportional data (i.e., a logistic regression) was used to determine the effect of location, treatment, and their interaction on fledging success, with Julian hatch day as a covariate. A Pearson's correlation test was used to examine the relationships between $\delta^{15}N$ and parasite abundance and glucose and hemoglobin levels.

Analyses were conducted with the lmer function (LMMs) and glm function (GLMs) using the lme4 package in R (Bates et al., 2014). Probability values were calculated using log-likelihood ratio tests using the Anova function in the car package (Fox & Weisberg, 2019). For one-way and two-way ANOVAs, we used type II and type III sum of squares, respectively. When we found a significant interaction between treatment and location, we used an estimated marginal means post-hoc test (Garofalo et al., 2022), using the function emmeans with the emmeans package in R (Lenth, 2021).

3 | RESULTS

3.1 | Effect of parasite treatment and urbanization on parasite load

Urban and non-urban finches initiated breeding in early February 2019. The first urban nest hatched approximately 7 days before the first non-urban nest. Although Julian hatch day was, on average, earlier for urban nests (mean ± SE: 86.24 ± 3.99 days) compared to non-urban nests (94.17 ± 4.07 days), Julian hatch day did not differ significantly between locations ($\chi^2 = 1.85$, $p = .17$). Furthermore, location, Julian hatch day, and their interaction did not significantly affect brood size (urban: 2.66 ± 0.18 nestlings, $n = 38$ nests; non-urban: 2.87 ± 0.17 nestlings, $n = 30$ nests; location: $\chi^2 = 0.44$, $p = .51$; hatch day: $\chi^2 = 0.68$, $p = .41$; interaction: $\chi^2 = 1.12$, $p = .29$). Eight nests were classified as depredated, either due to direct evidence of depredation (e.g., body parts found) or because they were likely depredated (e.g., all nestlings disappeared over a short period of time and prior to the typical fledging window).

The experimental treatment of nests with permethrin was nearly 100% effective at removing flies (Figure 1a); only one fumigated, urban nest had eight flies after the treatment. Within the sham-fumigated treatment, non-urban nests had significantly fewer flies than urban nests ($\chi^2 = 54.49$, $p < .0001$). Fourteen of 17 (82.35%) non-urban, sham-fumigated nests were naturally parasitized by 27.00 (±5.20) flies (Figure 1a). Seventeen of 21 (80.95%) urban, sham-fumigated nests were parasitized by 15.95 (±3.15) flies. One sham-fumigated nest disappeared due to predation after the nestlings were banded, and therefore we could not quantify parasitism. Julian hatch day did not significantly predict nest parasite abundance ($\chi^2 = 1.58$, $p = .21$).

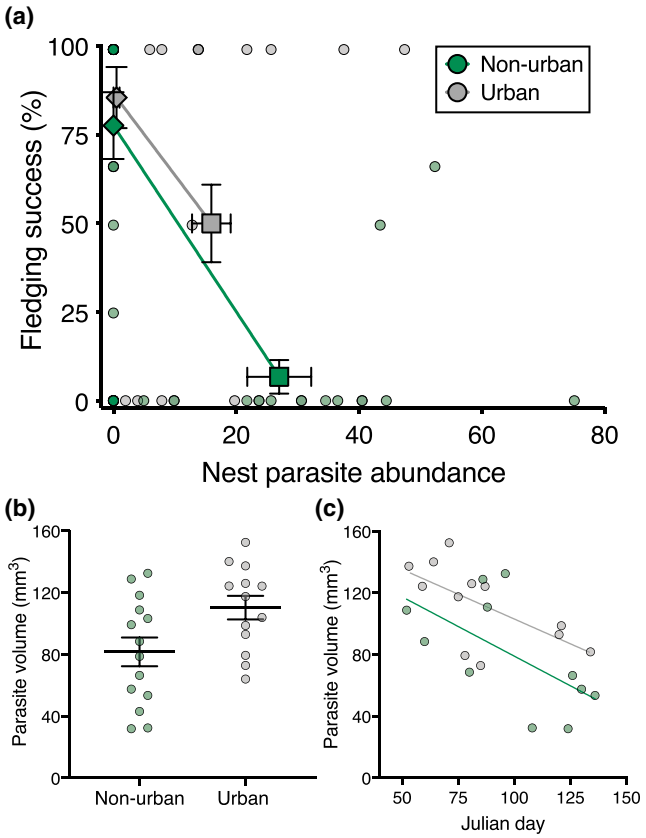

FIGURE 1 (a) The influence of urbanization on mean (±SE) nest parasite abundance (number of flies per nest) and % fledging success. Fledging success did not differ significantly for fumigated nests in the urban and non-urban locations. In sham-fumigated nests, urban nests had, on average, fewer flies and higher fledging success compared to non-urban nests. Each point represents an individual nest, and darker points indicate higher sample sizes. Diamonds represent the fumigated treatment, and squares represent the sham-fumigated treatment. (b) Pupal volume (mm^3) was larger in the urban location compared to the non-urban location. Each point represents a mean (±SE) volume for a nest. (c) Parasite volume decreased throughout the nesting season in both locations. Means and standard error bars are represented in (a, b).

Parasite volume was larger in urban nests compared to non-urban nests (Figure 1b; Table 1; $\chi^2 = 5.85$, $p = .02$). Urban fly pupae were, on average, 26% larger than non-urban fly pupae (urban: 100.30 ± 7.62 mm^3, non-urban: 81.61 ± 9.29 mm^3). This difference was not related to competition among flies within the nest because parasite intensity was not related to parasite volume ($\chi^2 = 0.01$, $p = .97$). Rather, Julian hatch day predicted parasite volume, with earlier nests having larger flies compared to later nests (Figure 1c; $\chi^2 = 11.01$, $p < .001$).

TABLE 1 Sample sizes for the effect of location (non-urban or urban) and parasite treatment (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 volume (mm^3)	NA	81.61 ± 9.29 (90/14)	NA	110.30 ± 7.62 (93/13)
$\delta^{15}N$ values	NA	8.81 ± 0.26 (11/11)	NA	13.05 ± 0.49 (7/7)
$\delta^{13}C$ values	NA	−20.60 ± 0.36 (11/11)	NA	−19.59 ± 0.79 (7/7)
C:N values	NA	3.98 ± 0.16 (11/11)	NA	4.01 ± 0.19 (7/7)
Individual nestling sample sizes: (# nestlings/# nests)				
Scaled mass index	8.93 ± 0.24 (27/12)	7.94 ± 0.40 (13/6)	8.72 ± 0.25 (37/15)	8.63 ± 0.33 (31/12)
Bill surface area	37.54 ± 2.14 (27/12)	33.55 ± 2.72 (13/6)	32.69 ± 1.97 (37/15)	38.00 ± 2.29 (31/12)
Tarsus length (mm)	16.73 ± 0.42 (27/12)	15.06 ± 0.76 (13/6)	15.76 ± 0.53 (37/15)	16.11 ± 0.67 (31/12)
Hemoglobin levels (g/dL)	9.83 ± 0.48 (10/10)	6.18 ± 0.67 (6/6)	9.15 ± 0.30 (18/14)	8.09 ± 0.74 (11/9)
Glucose levels (mg/dL)	277.00 ± 13.30 (16/12)	190.80 ± 23.42 (6/4)	232.40 ± 10.00 (18/12)	262.20 ± 19.22 (15/9)
$\delta^{15}N$ values	2.87 ± 0.16 (11/11)	1.90 ± 0.40 (2/2)	6.79 ± 0.43 (12/11)	7.31 ± 0.58 (9/8)
$\delta^{13}C$ values	−17.00 ± 0.78 (11/11)	−18.78 ± 0.33 (2/2)	−17.30 ± 0.89 (12/11)	19.04 ± 1.04 (9/8)
C:N values	10.30 ± 1.34 (11/11)	6.28 ± 0.63 (2/2)	7.19 ± 0.68 (12/11)	9.15 ± 1.15 (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%)

3.2 | Effect of location and landmarks on nesting and parasite abundance

Of all urban nests (sham-fumigated and fumigated), one nesting hotspot was identified within the urban location ($n = 37$ total nests; $n = 19$ hotspot nests), which was at the airport (Figure S1). The hotspot grid resulted in 14 cells (78 m × 78 m) with 90% confidence of being a hotspot, with *Z*-scores ranging between 2.90 and 6.02. The remaining cells in the study location were not identified as significant nesting hot spot or cool spot. Of the 19 nests within the nesting hotspot, only the sham-fumigated nests ($n = 9$) contained flies ($n = 7$), and all but one of these was located near a bench. Hotspots were not identified for fledging success, nor did we identify parasite hotspots among sham-fumigated nests.

3.3 | Effect of parasitism and urbanization on nestling health

The interaction between location and treatment and the main effect of location did not significantly affect body condition (i.e., scaled mass index; Table 1; interaction: $\chi^2 = 2.20$, $p = .14$; location: $\chi^2 = 0.36$, $p = .55$). However, nestlings from sham-fumigated nests had, on average, lower body condition than nestlings from fumigated nests ($\chi^2 = 4.01$, $p = .045$). The interaction between the main effects of treatment and location did not significantly affect bill surface area (Table 1; interaction: $\chi^2 = 3.09$, $p = .08$; treatment: $\chi^2 = 0.71$, $p = .40$; location: $\chi^2 = 0.13$, $p = .72$). However, most nestlings from non-urban, sham-fumigated nests died before they could be measured; only 13 (of 44) non-urban nestlings from six sham-fumigated nests survived to be measured.

The interaction between treatment and location affected hemoglobin levels ($\chi^2 = 6.16$, $p = .01$), with nestlings from non-urban, sham-fumigated nests having lower hemoglobin than nestlings from urban, sham-fumigated nests (estimate = −1.98 ± 0.85, $p = .03$) and non-urban, fumigated nests (Figure 2; Table 1; estimate = −3.65 ± 0.84, $p = .0001$). The main effect of location did not significantly affect nestling hemoglobin levels ($\chi^2 = 1.06$, $p = .30$). However, treatment influenced hemoglobin levels, with sham-fumigated nestlings having lower hemoglobin levels than fumigated nestlings ($\chi^2 = 18.82$, $p < .0001$).

The interaction between treatment and location influenced nestling glucose levels ($\chi^2 = 9.34$, $p = .002$), with nestlings from non-urban, fumigated nests having higher glucose levels than urban,

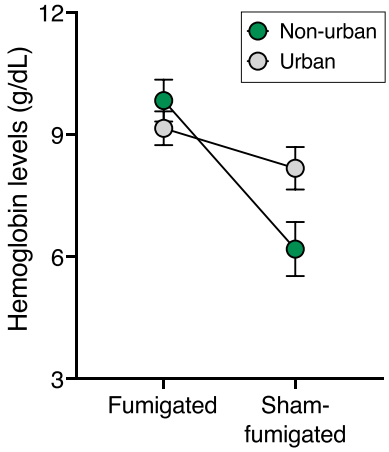

FIGURE 2 Effect of parasitism and urbanization on mean (±SE) blood loss (hemoglobin) in nestling finches. Nestlings from sham-fumigated nests had lower hemoglobin levels than nestlings from fumigated nests. Non-urban nestlings from sham-fumigated nests had lower hemoglobin levels than nestlings from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

fumigated nestlings (estimate = 47.40 ± 18.20, $p = .01$) and non-urban, sham-fumigated nestlings (Table 1; estimate = 80.20 ± 25.80, $p = .004$). The main effect of treatment affected glucose levels, with nestlings from fumigated nests having higher glucose levels than nestlings from sham-fumigated nests ($\chi^2 = 9.75$, $p = .002$). Location also affected glucose levels, with non-urban nestlings having higher glucose levels than urban nestlings ($\chi^2 = 6.87$, $p = .009$). Glucose levels were not correlated with hemoglobin levels ($r = .14$, $p = .45$).

The interaction between treatment and location affected fledging success ($\chi^2 = 8.28$, $p = .004$); the fledging success of fumigated nestlings did not differ between locations (estimate = −0.45 ± 0.56, $p = .41$), but for sham-fumigated nestlings, fledging was lower in non-urban locations compared to urban locations (Figure 1a; Table 1; estimate = −2.73 ± 0.63, $p = .001$). The main effect of treatment, but not location ($\chi^2 = 0.67$, $p = .41$), influenced fledging success with higher survival of nestlings from fumigated nests than sham-fumigated nestlings ($\chi^2 = 44.30$, $p < .0001$). The interaction between treatment and location and the main effect of treatment did not significantly affect fledging age (interaction: $\chi^2 = 0.10$, $p = .75$; treatment: $\chi^2 = 0.20$, $p = .66$), but location affected fledging age with urban nestlings left the nest approximately 1 day later than non-urban nestlings ($\chi^2 = 3.71$, $p = .05$).

3.4 | Effect of urbanization on finch and parasite diet

The interaction between sample type and location did not significantly affect $\delta^{15}N$ (Table 1; $\chi^2 = 0.00$, $p = .99$). However, flies were enriched in $\delta^{15}N$ compared to nestling feces ($\chi^2 = 164.94$, $p < .0001$), and all urban samples were enriched in $\delta^{15}N$ compared to non-urban samples (Figure 3a; $\chi^2 = 108.13$, $p < .0001$). Nest parasite abundance correlated negatively with $\delta^{15}N$ values (Figure 3b; $r = -.64$, $p = .002$).

The interaction between sample type and location and the main effect of location did not affect $\delta^{13}C$ (Table 1; interaction: $\chi^2 = 1.16$, $p = .28$; location: $\chi^2 = 0.43$, $p = .51$). However, nestling feces were enriched in $\delta^{13}C$ compared to flies ($\chi^2 = 11.21$, $p = .0008$). Similarly, the interaction between sample type and location and the main effect of location did not affect carbon to nitrogen ratio (C:N; Table 1; interaction: $\chi^2 = 0.74$, $p = .39$; location: $\chi^2 = 2.04$, $p = .15$), but nestling feces had a higher C:N compared to flies ($\chi^2 = 21.60$, $p < .0001$).

3.5 | Sequencing, quality control, and alignment

In total, 42 paired-end nestling libraries were constructed, including 11 urban sham-fumigated, five non-urban sham-fumigated, 14 urban fumigated, and 12 non-urban fumigated nestlings (1 nestling per nest). Altogether, the paired-reads post-QC ranged between 15.86 M and 42.16 M, and alignment rates against the *G. fortis* genome ranged from 80.74% to 91.81% (Table S1).

3.6 | Differentially expressed genes

A pairwise differential expression analysis observing two locations (urban and non-urban) and two treatments (fumigated and sham-fumigated), with an interaction, was conducted (File S1). A total of 5123 genes ($p_{adj} < .1$) were differentially expressed in sham-fumigated nestlings across the urban (upregulated) and non-urban (downregulated) locations, of which 2521 were upregulated and 2602 were downregulated. Altogether, 57 genes demonstrated strong expression patterns (±4-fold change), including frizzled class receptor 10 FZD10 (+16.20-fold change), a primary receptor for Wnt signaling, leucine rich repeat and Ig domain containing 3 LINGO3 (+5.35-fold change), which has been shown to regulate mucosal tissue regeneration in humans and promote wound healing, adenosine deaminase (ADA) (−8.17-fold change), associated with hemolytic anemia, and hepatic leukemia factor HLF (−5.64-fold change), known to influence the renewal of hematopoietic stem cells (Chen & Mitchell, 1994; Komorowska et al., 2017; Wang et al., 2016; Zullo et al., 2021). In fumigated nestlings, only two genes were upregulated in the urban location: bisphosphoglycerate mutase BPGM (+3.24-fold change), a regulator of erythrocyte metabolism and hemoglobin in red blood cells, and zinc finger protein GLIS1 (+3.40-fold change), which has been significantly associated with bill length (Lundregan et al., 2018; Xu et al., 2020). There were no significantly downregulated genes.

When comparing sham-fumigated versus fumigated nestlings in the urban location, a total of 768 genes were differentially expressed, with 570 being upregulated in sham-fumigated nestlings and 198 being downregulated. The vast majority of genes showed moderate-to-low fold-change patterns, with only three exhibiting strong expression patterns: inorganic pyrophosphate transport regulator ANKH (+4.43-fold change), prokineticin 2 PROK2 (+4.38-fold change), and plexin A2 PLXNA2 (−4.08-fold change).

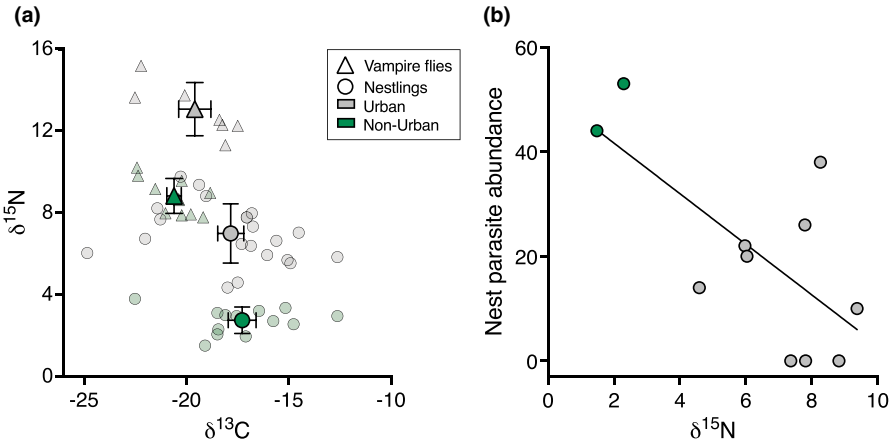

FIGURE 3 (a) Mean (±SE) $\delta^{15}N$ and $\delta^{13}C$ values of nestlings and flies in the urban and non-urban locations. Urban individuals were enriched with $\delta^{15}N$ compared to non-urban individuals. Overall, flies were enriched with $\delta^{15}N$ compared to nestlings, and nestlings were enriched with $\delta^{13}C$ compared to flies. (b) Nest parasite abundance (number of flies per nest) was negatively correlated with $\delta^{15}N$ values. Each point represents an individual.

By comparison, sham-fumigated versus fumigated nestlings from the non-urban location yielded 7064 differentially expressed genes, 3709 of which were upregulated in sham-fumigated nestlings and 3355 downregulated. Again, the majority of genes exhibited moderate-to-low fold change, with only 314 displaying strong expression patterns. This included an assembly factor for spindle microtubules, ASPM (+6.34-fold change), which has an apparent role in neurogenesis and neuronal development (Nam et al., 2010) (Figures S3 and S4).

3.7 | Gene enrichment related to host defense mechanisms

The gene enrichment of sham-fumigated nestlings from the urban and non-urban locations was compared to identify potential host defense mechanisms to explain the expression patterns observed. Analysis of the enriched *immune system process* GO terms revealed 21 significantly ($p < .05$) upregulated terms (urban) and 12 significantly downregulated terms (non-urban) categorized by resistance (adaptive and innate) and tolerance (File S2).

First, we examined enrichment patterns related to *adaptive immunological resistance* (Figure 4a). The urban sham-fumigated nestlings exhibited strong enrichment of lymphocyte differentiation ($p < .02$) and T-cell pathways related to regulation of CD4-positive, alpha-beta T-cell activation ($p < .01$) and NK T-cell differentiation ($p < .05$). By comparison, non-urban sham-fumigated nestlings yielded 21 significant adaptive immunity terms. Of those, 10 were related to Ig antibodies, including positive regulation of isotype switching to IgG isotypes ($p < .001$) and somatic hypermutation of immunoglobulin genes ($p < .001$). The rest related to B-cell proliferation ($p < .01$) and activation ($p < .05$), leukocyte differentiation ($p < .04$) and proliferation ($p < .05$), regulation of antigen receptor-mediated signaling pathways ($p < .04$), and germinal center formation ($p < .01$).

We next examined enrichment patterns related to *innate immunological resistance* (Figure 4b). Urban sham-fumigated nestlings were enriched for both type I interferon (IFN)-mediated signaling ($p < .03$) and toll-like receptor 9 signaling ($p < .05$). Among the non-urban nestlings, only negative regulation of the innate immune response ($p < .07$) was enriched. Finally, we examined enrichment related to *tolerance* (Figure 4c). Urban sham-fumigated nestlings had three significantly enriched terms specific to red blood cells, including erythrocyte differentiation ($p < .003$) and development ($p < .002$), as well as enucleate erythrocyte differentiation ($p < .01$). Among non-urban nestlings, regulation of megakaryocyte differentiation yielded a *p*-value less than .07.

3.8 | Co-expression analysis

A differential co-expression analysis was conducted to provide novel insights on other traits that may explain gene expression patterns in nestling finches. Correlations in transcript levels across 12 traits (urban, sham-fumigated, hemoglobin level, glucose level, alive, $\delta^{13}C$, $\delta^{15}N$, C:N, days old, parasite abundance, bill surface area, and scaled mass index) introduced 44 modules (File S3; Figures S5–S8). Nestling fledging success ("alive" trait) presented the most significant correlations across the gene set, as illustrated by module A ($r = .67$, $p < .0001$) and module B ($r = -.82$, $p < .0001$), respectively (Figure 5). From the 2338 total genes included in module A, 43 yielded 19 significantly enriched *immune system process* terms related to T-cell activation, lymphocyte differentiation, and negative regulation of hemopoiesis. Comparatively, 55 of the 3422 genes in module B activated 21 immune pathways related to somatic hypermutation of immunoglobulin genes, leukocyte-mediated cytotoxicity, and, interestingly, erythrocyte differentiation.

Apart from mortality, other traits were strongly correlated with modules A and B. Specifically, module A was negatively correlated with parasitism ($r = -.59$, $p < .0001$) and nest parasite abundance ($r = -.74$, $p < .0001$), and positively correlated with hemoglobin level ($r = .66$, $p < .0001$), glucose level ($r = .48$, $p < .001$), and $\delta^{13}C$ ($r = .32$, $p < .04$). Specifically, module B was positively correlated with parasitism ($r = .57$, $p < .0001$) and nest parasite abundance ($r = .62$, $p < .0001$), and negatively correlated with hemoglobin level ($r = -.52$, $p < .0004$), glucose level ($r = -.55$, $p < .0002$), $\delta^{13}C$ ($r = -.34$, $p < .03$), and scaled mass ($r = -.45$, $p < .003$). By comparison, although there

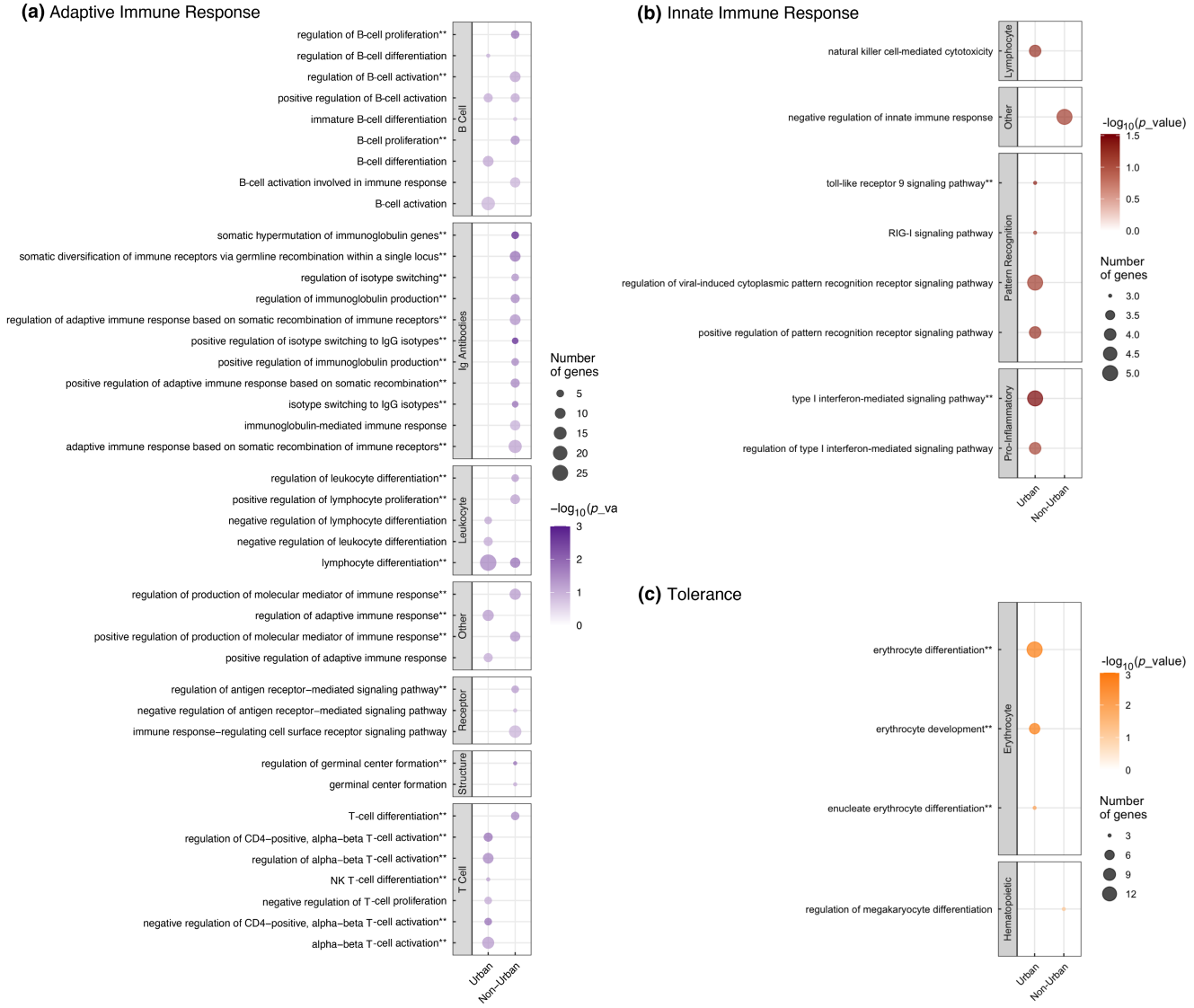

FIGURE 4 Gene Ontology enrichment analysis of sham-fumigated urban (upregulated) and non-urban (downregulated) differentially expressed genes utilizing ClueGO. (a) The adaptive immune response functional categories shown in purple were partitioned into seven sub-categories: B cell, Ig antibodies, leukocyte, receptor, structure, T cell, and other. (b) The innate immune response categories, shown in red, were divided into four sub-categories: lymphocyte, pattern recognition, pro-inflammatory, and other. (c) Tolerance categories, shown in orange, were split into two sub-categories: erythrocyte and hematopoietic. Log_{10} (p-value) significance of unique and shared terms is depicted as a color saturation gradient (** $p < .05$), and the number of genes supporting each ontology term is represented by circle size.

were no notable correlations to urbanization in the described modules, the enriched immune pathways better explain patterns of expression observed in urban and non-urban sham-fumigated finches.

4 | DISCUSSION

Our study found that urban living partially rescues nestling small ground finches from the lethal effects of parasitism by invasive avian vampire flies. Fledging success of sham-fumigated nestlings did not differ significantly between locations, which suggests that urbanization alone does not affect fledging success. On average, urban nests had significantly fewer flies than non-urban nests, but even urban nestlings from nests with high parasite abundances survived. These results indicate that urban nestlings have effective resistance and tolerance mechanisms to deal with avian vampire flies. Differences in diet (measured using stable isotopes) between locations could explain why urban nestlings see fewer negative fitness consequences, but these results are correlative. Gene expression analyses revealed that resistance and tolerance mechanisms might underlie differences in parasite effects. Sham-fumigated urban nestlings differentially expressed genes within pathways associated with tolerance and innate immunological resistance, compared to sham-fumigated non-urban nestlings. In contrast, sham-fumigated non-urban nestlings differentially expressed genes within pathways associated with adaptive immunological resistance. The gene expression results

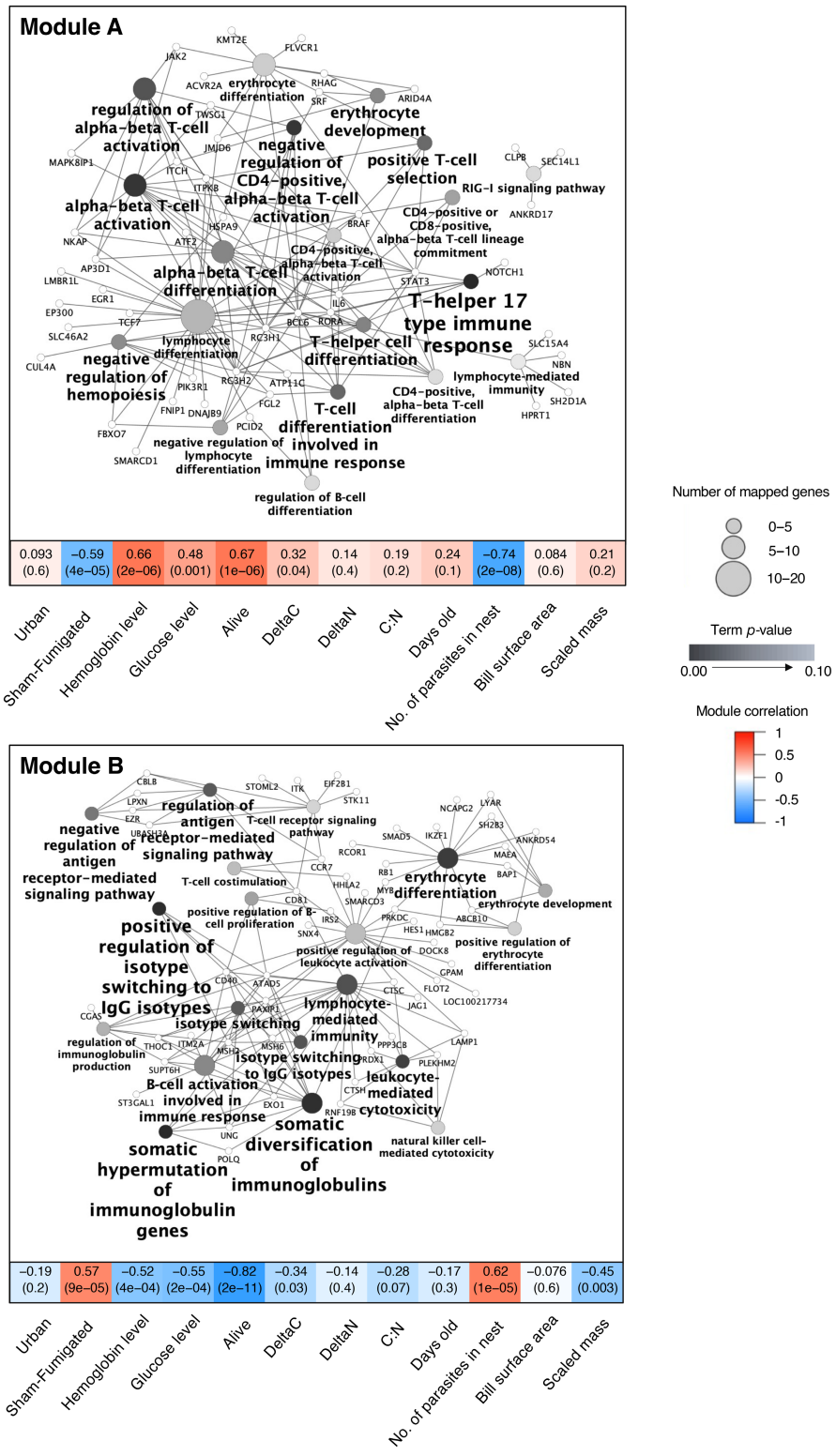

FIGURE 5 Gene Ontology enrichment network of co-expression modules A (top) and B (bottom). WGCNA trait correlation is depicted on a scale from 0 (white) to 1 (red), and negative trait correlation significance is from 0 (white) to −1 (blue). WGCNA *p*-values are shown in parentheses. The co-expressed genes are represented with ClueGO in pathway form. The significance of enriched Gene Ontology terms is represented by the gray gradient. The size of the circle represents the number of genes associated with the enriched term.

suggest that non-urban nestlings are investing in adaptive immunity, but that this type of response is not an effective defense mechanism against the parasite since few nestlings survived.

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

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

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

Although sham-fumigated non-urban nestlings did not exhibit significantly enriched innate immune pathways, they did differentially express genes involved in pathways related to the adaptive immune response. Specifically, T cell, B cell, and Ig pathways were expressed, but 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 fledging success compared to non-urban nestlings is that resistance is heightened when the innate and adaptive immune systems 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 et al., 2014; Howick & Lazzaro, 2014; Lochmiller & Deerenberg, 2000; Sheldon & Verhulst, 1996; Sternberg et al., 2012; Svensson et al., 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 locations in the Galápagos provide a reliable source of human food for animals, including finches (De León et al., 2019). For example, some restaurants have outdoor dining, where food falls off tables and finches feed on the tables (Figure S9). Our stable isotope results corroborate the idea that diet differs between urban and non-urban nestlings. In fact, higher δ^{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 δ^{15}N values were 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 & Kyriazakis, 2001). Although our results suggest that urban finches are more physiologically resistant to flies than non-urban finches, another potential explanation is that nest material affects the fledging success 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 (Suárez-Rodríguez 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% fledging success despite high parasite abundances (up to 48 flies), indicating that these urban nestlings are tolerant of flies. Studies have demonstrated that low finch fledging success 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 locations. 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 & 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 enough erythropoietin. Urban nestlings have higher δ^{15}N values, which is likely because they are feeding on more iron-rich meat products than non-urban birds. Thus, this difference in diet could explain the increased tolerance related to red blood cell recovery but requires further studies on iron and nestling endocrinology. Finally, although non-urban nestlings did not exhibit any

significantly enriched pathways related to tolerance, we found that ADA was significantly upregulated. Interestingly, overexpression of ADA in red blood cells has been shown to cause hemolytic anemia (Chen & Mitchell, 1994), which could be additionally responsible for the low fledging success of non-urban finches.

Blood glucose levels were lower in non-urban nestlings from sham-fumigated nests compared to other nestlings. One explanation is that blood loss affects the production of glucose, but few studies have examined this relationship (Minias, 2015). Minias (2014) found a negative correlation between hemoglobin levels and glucose, likely because hyperglycemia-induced oxidative stress reduces the synthesis of the hormone erythropoietin (Winkler et al., 1999). This explanation is unlikely because, regardless of the direction of the relationship, nestling hemoglobin levels did not correlate significantly with glucose levels. Glucose is often studied in the context of insufficient nutrient intake, which can induce gluconeogenesis that converts stored glycogen to glucose, thereby increasing plasma glucose levels (Braun & Sweazea, 2008; Sweazea, 2022). Given this pattern, the non-urban sham-fumigated nestlings are predicted to have higher glucose levels than the other nestlings, which was not found in our study. Most of these nestlings died within days of sampling, and therefore they were unlikely to have any stored glycogen to convert to glucose, which is the most plausible explanation for our glucose results.

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

Heterogeneity across different urban locations could also impact finch-fly interactions on other islands in the Galápagos. Due to logistical limitations, replication of locations was not possible across islands. However, replication of locations would be helpful to understand whether context dependency exists across different urban locations. The Galápagos Islands have two other major towns, including Puerto Ayora of Santa Cruz Island with approximately 12,000 human residents and Puerto Villamil of Isabela Island with approximately 2000 human residents (Guerrero et al., 2017). Human population size often scales with anthropogenic features (e.g., artificial light at night, density of restaurants, number of vehicles) in urban locations, which can proportionally affect bird populations (reviewed in Isaksson, 2018). Thus, replicating our study in towns on other islands in the Galápagos could provide insight into whether the scale of urbanization impacts finch-fly interactions differently.

Naïve hosts are thought to lack defenses against novel parasites, which can cause declines and extinctions (Atkinson & LaPointe, 2009; Daszak et al., 2000; Keesing et al., 2010). Over the past decade, studies have found that invasive avian vampire flies can cause up to 100% mortality in Darwin's finches across islands in the Galápagos (Addesso et al., 2020; Kleindorfer & Dudaniec, 2016; Knutie et al., 2016; Koop et al., 2011; Koop, Le Bohec, et al., 2013; Koop, Owen, et al., 2013; McNew & Clayton, 2018; O'Connor et al., 2010, 2014). If humans cannot effectively control the fly (e.g., Knutie et al., 2014) or hosts do not evolve or develop defenses, some species may even go extinct (Fessl et al., 2010; Koop et al., 2016; Koop, Owen, et al., 2013). However, our study provides experimental evidence that the urban population of finches is relatively well-defended against avian vampire flies. Indeed, additional studies of urban and non-urban populations across years are needed to determine the magnitude of the urban effect, but preliminary data from other years (2022, 2023) and populations (Puerto Chino, San Cristobal) suggests that this pattern is observed beyond our study (SAK prelim. data). To be clear, we are not suggesting that the Galápagos Islands should be urbanized to increase defense against flies. Instead, the goal of our study is to demonstrate that some finches can defend themselves against the flies through various mechanisms, which can provide insight into management strategies (Ohmer et al., 2021).

AUTHOR CONTRIBUTIONS

Sarah A. Knutie: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; validation; visualization; writing – original draft; writing – review and editing. **Cynthia N. Webster:** Data curation; formal analysis; investigation; methodology; visualization; writing – original draft; writing – review and editing. **Grace J. Vaziri:** Data curation; funding acquisition; investigation; methodology; writing – original draft; writing – review and editing. **Lauren Albert:** Investigation; writing – review and editing. **Johanna A. Harvey:** Investigation; writing – review and editing. **Michelle LaRue:** Formal analysis; investigation; methodology; writing – original draft; writing – review and editing. **Taylor B. Verrett:** Investigation; writing – review and editing. **Alexandria Soldo:** Investigation; visualization; writing – review and editing. **Jennifer A. H. Koop:** Investigation; writing – review and editing. **Jaime A. Chaves:** Investigation; project administration; writing – review and editing. **Jill L. Wegrzyn:** Formal analysis; funding acquisition; investigation; methodology; supervision; visualization; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

Supplementary Files S1–S3 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: https://doi.org/10.6084/m9.figshare.24915045), and sequences have been uploaded to GenBank (BioProject accession number: PRJNA930453).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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