# A field-based quantitative analysis of sublethal effects of air pollution on pollinators

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**While the impact of air pollution on human health is well studied, mechanistic impacts of air pollution on wild systems, including those providing essential ecosystem services, are largely unknown, but directly impact our health and well-being. India is the world's largest fruit producer, second most populous country, and contains 9 of the world's 10 most polluted cities. Here, we sampled Giant Asian honey bees, *Apis dorsata*, at locations with varying air pollution levels in Bangalore, India. We observed significant correlations between increased respirable suspended particulate matter (RSPM) deposition and changes in bee survival, flower visitation, heart rate, hemocyte levels, and expression of genes related to lipid metabolism, stress, and immunity. Lab-reared *Drosophila melanogaster* exposed to these same sites also exhibited similar molecular and physiological differences. Our study offers a quantitative analysis on the current impacts of air pollution on insects, and indicates the urgency for more nonhuman studies to accurately assess the effects of pollution on our natural world.**

apis | behavioral ecology | ecosystem services | insect physiology | transcriptomics

Rapid urbanization, intense agriculture, and poor emission control and enforcement coupled with a dependence on 'old and poorly maintained vehicles' (1) over the past century have led to immense increases in air pollution in the developing world. According to the World Health Organization (WHO) recent estimates, India now contains 9 of the world's 10 most polluted cities (2). India also serves as the world's largest fruit and second-largest vegetable producer (3). Animal pollination is responsible for 35% of all crop productivity (4) and contributes significantly to the productivity of at least 75% of our global crop species (4, 5). Insects, and particularly bees, provide the majority of this pollination (4). Recent studies have documented the cataclysmic decline of insect populations across the world (6–8), and have suggested that reductions in habitat quality, including pollution, contribute to this loss (6, 8). One in six species of bees are also reported locally extinct in various regions throughout the world (8). However, few studies have considered the molecular and physiological impacts of air pollution on organisms other than humans that are equally exposed to airborne toxins such as respirable suspended particulate matter [RSPM; a complex mixture of particles less than 10 or 2.5 μM in diameter respirable by humans (9)] ground-level ozone ($O_3$), nitrogen dioxide ($NO_2$), sulfur dioxide ($SO_2$), and volatile organic compounds (10).

A number of studies over the past 40 y have acknowledged the impact of air pollution on insect populations and olfactory behavior. Effects of air pollution on insects include both direct toxicity and indirect decline due to disequilibria with higher or lower trophic levels (11). In some cases, air pollutants such as sulfur and nitrogen dioxide may increase the prevalence of insect pests such as aphids, perhaps due to a loss of natural enemies (12, 13). Pollutants can inhibit the search behavior of natural enemies through direct impacts on sensory physiology and behavior (14). For insects, air pollutants are likely to impact olfactory-guided behavior strongly, as chemosensory information is used in almost every aspect of insect biology from locating food, mates, and host sites, to avoiding predators (15). For example, the transport of chemical signals in the air used by pollinators to locate flowers and other organisms may be hindered or even destroyed by chemical reactions with pollutants (16). Confounding airborne particles, including smoke, have been shown to decrease bee alarm pheromone detection (17). Interestingly, a recent study implies that despite the potential exposure to higher air pollution levels, bumblebees in the UK can exploit resources in the city more effectively than in agricultural areas (18). The authors suggest this could be due to the reduced pesticide use in comparison to agricultural areas, and that urban areas may provide a refuge for pollinator populations.

Nevertheless, few studies have examined the underlying molecular and physiological correlation of air pollution on wild systems. For humans, air pollution has been associated with respiratory issues such as asthma (19) and lung cancer (20), circulatory impacts including ventricular hypertrophy (20) and heart disease, (21) sensory and neurological disorders such as retinopathy (22), Alzheimer's and Parkinson's diseases (21), autism (23), cognitive performance (24), and general effects associated with stress, toxicity, homeostasis, or immunity including

## Significance

**India is the world's largest fruit producer and second most populous country. Pollinators are therefore important for India's food security. India also contains 9 of the world's 10 most polluted cities, but the impact of air pollution on plant and animal systems is largely unknown. We performed a multiyear study in the megacity of Bangalore to correlate the mechanistic effects of air pollution on a major Indian pollinator, the Giant Asian honey bee, *Apis dorsata*. Wild honey bees and lab-reared *Drosophila* exposed to air pollution exhibited differences in survival, behavior, heart rate, blood cell count, and/or the expression of genes related to stress, immunity, and metabolism. Our study indicates the urgency for more studies on wild systems to better inform international air quality guidelines.**

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The authors declare no competing interest.

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obesity (21), fetal growth impairment (25), low birth weight (26), deaths, disease burden, and life expectancy (27), among many others. As a first principle, one could initially hypothesize that insects might also exhibit similar respiratory, circulatory, neurological, stress, homeostatic and/or immune system impacts upon exposure to air pollution, in addition to the behavioral and population level impacts already observed in previous insect studies.

Here, we have developed a field-based analysis to correlate the impact of RSPM on wild pollinator behavior, respiratory and circulatory physiology, immunology, and expression of genes related to stress, toxicity, homeostasis, and immunity as suggested by current insect behavior and human health studies. To this end, we examined the impact of air pollution on insect pollinators by sampling *Apis dorsata*, the Giant Asian honey bee, at distinct locations in the megacity of Bangalore, India [Fig. 1*A*; current population: 13.1 million (28)]. Bangalore has witnessed a 47% population increase in just 10 years. The measured RSPM values from 2015 to 2016 have exceeded the national permissible limit [60.0 μg/m$^3$, which is defined by the Ministry of Environment and Forest (MoEF), Government of India] (29) in 15 locations of Bangalore (30). *A. dorsata* (Fig. 1*B*) produces more than 80% of the honey in India and has been found to pollinate more than 687 plant species (31). *A. dorsata* is a rock bee often found nesting in cities. This species has been shown to migrate its colonies repeatedly over many kilometers throughout the year among urban, rural, and forest habitats (31, 32). As a consequence, any long-term physiological or behavioral impacts of urban air pollution will also impact the pollination services these insects provide to agricultural and forest areas where they migrate. *A. dorsata* is a rarely managed species (33), and there are no current beekeeping efforts in Bangalore. Coupled with its agricultural and ecosystems relevance, its common occurrence in cities makes it an excellent species to evaluate the impact of urban air pollution on wild systems and ecosystem services. Here, we correlate air pollution levels at various sites in Bangalore with differences in *A. dorsata* survival, floral visitation behavior, and cellular and molecular physiology including circulatory, immune, and gene expression aspects as suggested by human studies and known insect toxins such as pesticides. To control for potential impacts of diet and colony variation, and to determine if these responses could be generalized to other insect species, we also exposed laboratory-reared and age-matched *Drosophila melanogaster* to the same sites and performed the same physiological and molecular experiments.

## Results and Discussion

**Site Selection, Floral Visitation, and Survival.** Collection and observation sites were chosen as representative of varying pollution levels within a maximum linear distance of approximately 21 km between the two most distant sites. Each location was within 8 km of the next closest site (Fig. 1*A*). The RSPM (measured directly here as $PM_{10}$, particles < 10 μM) averaged 28.32 ± 10.51 (mean ± SD, $n$ = 78 measurement days) at rural (R), 33.73 ± 11.03 at low (L, $n$ = 115), 45.95 ± 20.52 at moderate (M, $n$ = 103), and 98.59 ± 55.43 μg/m$^3$ at the highly polluted site (H, $n$ = 118) between January 2017 to April 2019 (Fig. 1*C* and *SI Appendix*, Fig. S1*A*; Welch ANOVA test followed by Dunnett's T3 multiple comparisons test). The specific sampling locations were chosen to reduce the effects of other potential variables such as pesticide use, immediate floral abundance within 100 m of the collection site, traffic, shade, presence of human-made structures, and presence of Giant Asian honey bee colonies (*SI Appendix*, Table S1). As a proxy for general floral abundance encompassing the maximum foraging range of *A. dorsata* at each site (up to 3.5-km radius) (32, 35), we also analyzed variation in the Normalized Difference Vegetative Index (NDVI) concurrent with experimental dates (36–38 and *SI Appendix*, Fig. S1). At each location, we identified the [nonnative (39)] flowering tree, *Tecoma stans*, a very common ornamental plant present across India which blooms throughout the year and is pollinated by *A. dorsata*. We therefore choose this flowering plant as our species of interest for *A. dorsata* visitation behavior, and made all observations and collections during morning bee visitation times for this species (40). At each site, we observed multiple colonies (*SI Appendix*, Table S1), but could not assign collected bees to specific colonies. Given that *A. dorsata* frequently migrate their colonies (31, 32), it is highly unlikely that the same colonies were present at each site across the entire 3-y study (*SI Appendix*, Table S2). Therefore, to reduce the potential for pseudoreplication from specific colonies as well as effects of diet and colony variation within and between sites, we randomly collected bees while they were foraging at *T. stans* flowers at multiple sites over several time points throughout the study (*SI Appendix*, Table S2). Multiple sites

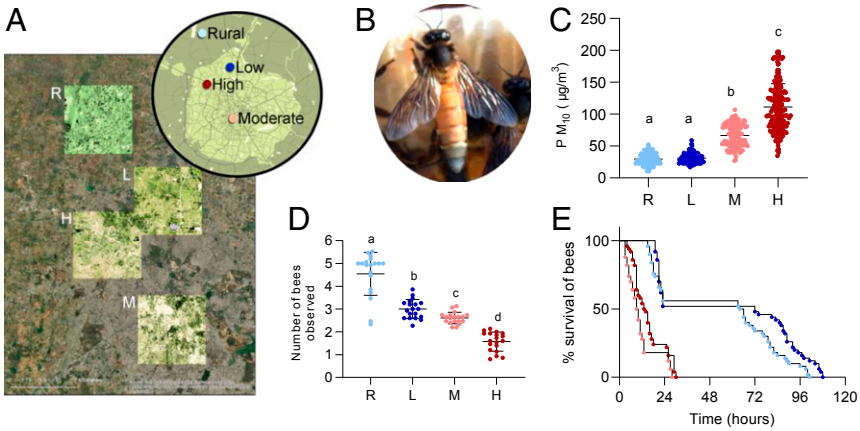



**Fig. 1.** Floral visitation and survival studies in the Giant Asian honey bee. (*A*) Study area and chosen sites (34). (*B*) Study animal, *A. dorsata*, the Giant Asian honey bee. (*C*) $PM_{10}$ measurements in μg/m$^3$ over the 3-y study period. [R, $n$ = 78; L, $n$ = 115; M, $n$ = 103; H, $n$ = 118; Welch's F (3, 215.3) = 67.94, $P$ < 0.0001, Cohen's d (L vs. H) = 1.55] (*D*) Average number of bees per day observed foraging from 20 *T. stans* inflorescences over 5 min [$n$ = 400 inflorescences/site; Welch's F (3, 37.63) = 67.91, $P$ < 0.0001, Cohen's d (L vs. H) = 3.41]. (*C* and *E*) Welch ANOVA test followed by Dunnett's T3 multiple comparisons test. See *SI Appendix*, Tables S5 and S6 for multiple comparison statistics. (*E*) Kaplan–Meier survival curves with Log-rank (Mantel–Cox) test for percent survival of fed bees under laboratory conditions after 24 h ($n$ = 50/site; $\chi^2$ = 127.7, df; 3, $P$ < 0.0001). Series with different letters denote significant differences (Welch ANOVA test followed by Dunnett's T3 multiple comparisons test, $P$ < 0.05). R = rural, L = low, M = moderate, and H = highly polluted site. Scatter dot plots with error bars represent mean ± SD.

were visited within the same time period to mitigate weather or seasonal concerns. In total, we have sampled 1,820 Giant Asian honey bees over 3 y ($n$ = 455 bees/site).

The number of bees per inflorescence (5 min observation of 20 inflorescences per 20 d; $n$ = 1,600, 400 inflorescences/site) was significantly different between sites with varying pollution levels (Fig. 1*D*; Welch ANOVA test followed by Dunnett's T3 multiple comparisons test) even though the number of flowers in the least polluted rural site was less (*SI Appendix*, Fig. S1*D*; $n$ = 4,000, 1,000/site, Welch ANOVA test followed by Dunnett's T3 multiple comparisons test). This is in agreement with previous studies correlating population levels or pollinator behavior with RSPM levels (16, 17). Temperature, humidity, and wind speed did not differ between sites over the time periods where bees were observed or collected, and NDVI was significantly higher only in the rural site, while the other three sites did not differ in

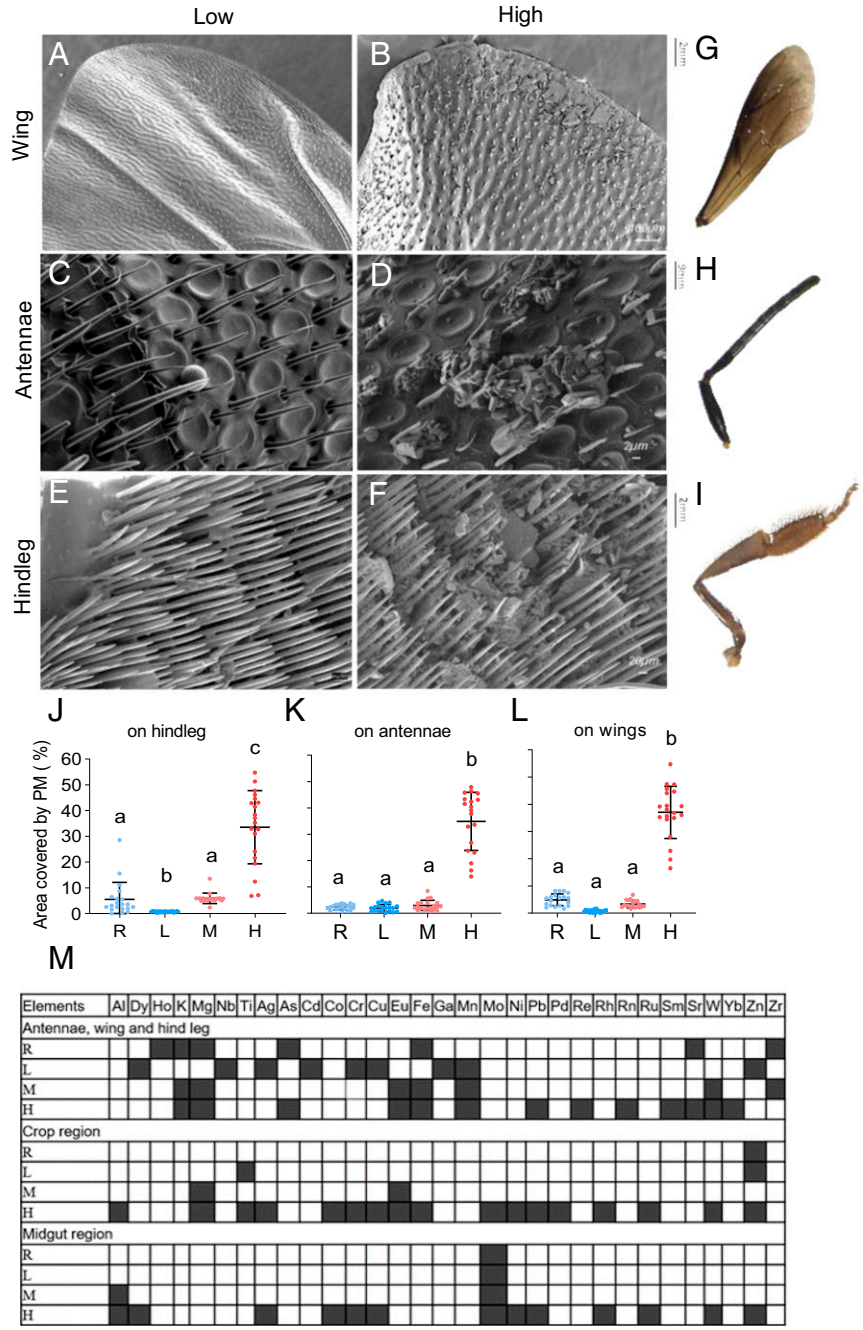



**Fig. 2.** Morphology and toxicology in the Giant Asian honey bee. SEM images of Giant Asian honey bee appendages bearing RSPM from low and high sites. [(*A* and *B*) bar = 100 μM] portion of wings. [(*C* and *D*) bar = 2 μM] geniculate antennal section. [(*E* and *F*) bar = 20 μM] section of hindleg (3,650× magnification for antennae and legs, 350× magnification for wings). (*G–I*) Light microscopic images of wing (bar = 2 mm), antennae (bar = 9 mm), and metathoracic leg (bar = 2 mm), respectively (10× magnification). Percentage area covered by RSPM ($n$ = 480, 40 samples/section/site) on hindleg [*J*; Welch's F (3, 31.89) = 80.59, $P$ < 0.0001, Cohen's d (L vs. H) = 3.27)], antennae [*K*; Welch's F (3, 38.46) = 53.87, $P$ < 0.0001, Cohen's d (L vs. H) = 4.16] and wing [*L*; Welch's F (3, 33.66) = 126.4, $P$ < 0.0001, Cohen's d (L vs. H) = 4.16]. (*M*) Summary of metals detected (black for each element and site) on each body segment using SEM-EDX. Series with different letters denote significant differences (Welch ANOVA test followed by Dunnett's T3 multiple comparisons test, $P$ < 0.05). See *SI Appendix*, Tables S5 and S6 for multiple comparison statistics. R = rural, L = low, M = moderate, and H = highly polluted site. Scatter dot plots with error bars represent mean ± SD.

vegetative index over the same period (*SI Appendix*, Fig. S1 *A–C*; $n = 200$ samples per site for temperature, humidity, and windspeed, $n = 16$ per site for NDVI, Welch ANOVA test followed by Dunnett's T3 multiple comparisons test). Giant Asian honey bees collected from moderate and highly polluted sites exhibited significantly lower survival rates over the total observation period (Fig. 1*E*; $n = 200$, 50 bees/site; $\chi^2$ test), despite providing 10% honey water ad libitum and removing bees that did not feed and died within the first 4 h. We note that this difference is not likely due to extreme age differences as no bees from any site exhibited wing damage generally observed during senescence (41) (*SI Appendix*, Fig. S1*L*). Collectively, the field studies suggest a correlation between RSPM levels and *A. dorsata* behavior and survival. We thus continued to examine underlying physiological and molecular correlates to these pollution levels.

**Morphology and Toxicology.** We next examined if there was evidence of air pollution on the exoskeleton and internal tissues of field-collected Giant Asian honey bees from each site. Previous studies have shown increased deposition of particulate matter (RSPM) on bee epicuticular waxy layers corresponding to higher air pollution levels (42). First, we observed no difference in the total weight (*SI Appendix*, Fig. S1*K*; $n = 120$, 30 bees/site; Welch ANOVA test followed by Dunnett's T3 multiple comparisons test) and morphometric measurements of hindlegs, antennae, wings, and intertegular spans (43) for bees collected from the four sites (*SI Appendix*, Fig. S1 *F–I*; $n = 80$, 20 bees/site, Welch ANOVA test followed by Dunnett's T3 multiple comparisons test). We then quantified the deposition of RSPM by measuring the percentage area covered by particulate matter on the outermost layer [0.2 to 0.4 μm thick epicuticular waxes (44)] for hindlegs, antennae, and wings with scanning electron microscopy (SEM; $n = 480$, 120 sections/site), after allowing bees to groom (45, 46). We observed RSPM present on the edges and upper surface of the wings (Fig. 2 *A*, *B*, and *G*). Antennae displayed RSPM deposition near the tip when compared to the rest of the segments (Fig. 2 *C*, *D*, and *H*), and the inner and outer cuticular layer of hindlegs also showed congestion of particles (Fig. 2 *E*, *F*, and *I*).

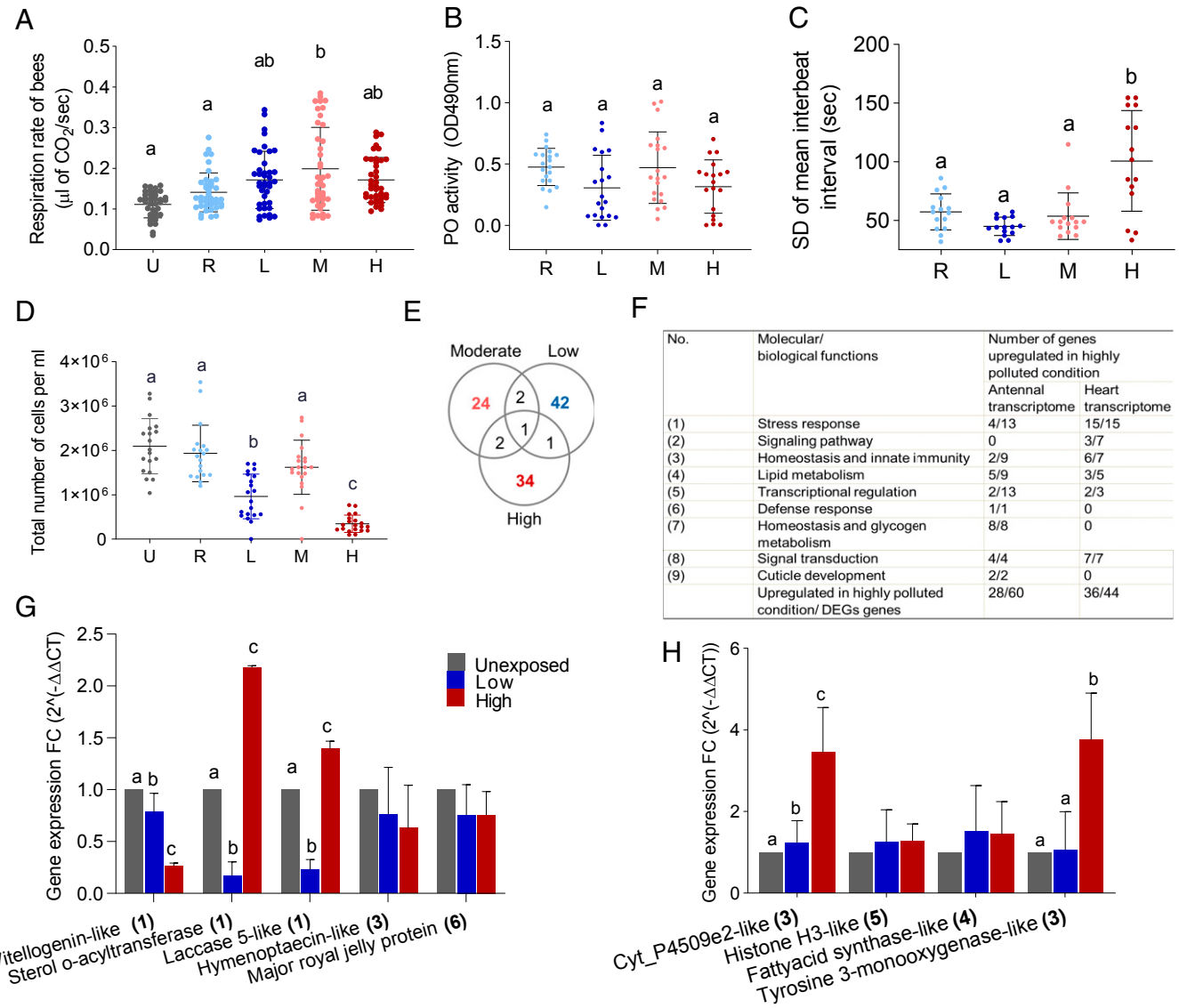
Overall, the percent area covered with RSPM for all three selected body sections (Fig. 2 *J–L*) was significantly higher for bees collected from the highly polluted site ($n = 480$ total sections, 40 samples/section/site, 20 bees/site; Welch ANOVA test followed by Dunnett's T3 multiple comparisons test). To determine the elemental composition of this RSPM, we coupled SEM with X-ray spectroscopy (SEM-EDX) analysis ($n = 80$ bees, 20/site), which revealed the presence of several heavy metals including arsenic (As), lead (Pb), tungsten (W), aluminum (Al), and several other metals, with the highest number of metallic elements present on bees collected from the highly polluted site (Fig. 2*M*), as in ref. 42. Several of these metals have been shown to be toxic for insects, invertebrates, and humans in a number of studies (47, 48). This provided our first direct evidence of increased exposure to pollutants and known toxins correlating with increased RSPM levels in our field sites.

***Respiratory and circulatory physiology and immunology.*** As suggested by human health studies, we then examined other aspects of bee physiology, including respiratory and circulatory physiology, and immunology. Respiration rates of Giant Asian honey bees collected from all four sites showed few significant differences between sites [Fig. 3*A*, $n = 200$, 40 bees/site including unexposed bees (3- to 5-d-old bees maintained in the incubator at 33 °C), Welch ANOVA test followed by Dunnett's T3 multiple comparisons test]. Phenoloxidase activity, which has been shown to initiate the humoral and cellular immune response against pathogens (49), also did not differ in bees between sites (Fig. 3*B*; $n = 80$, 20/site, Welch ANOVA test followed by Dunnett's T3 multiple comparisons test). However, while the mean heart rate itself did not differ between bees from different sites (*SI Appendix*, Fig. S1*J*; $n = 15$ bees/site), the mean SD of interbeat interval of heart rate (IBI), an alternate method and an indicator of arrhythmicity (50), was significantly different for bees collected from the highly polluted site (Fig. 3*C*, *SI Appendix*, Fig. S4*D*, and Movie S1; $n = 180$, 450 sections/site with 30 sections/bee; Welch ANOVA test followed by Dunnett's T3 multiple comparisons test). Total hemocyte count (THC) was also significantly different for bees from the low and highly polluted sites (Fig. 3*D*; $n = 100$, 20 bees/site including unexposed bees, Welch ANOVA test followed by Dunnett's T3 multiple comparisons test). Together, these results suggest a correlation between increased RSPM and impacts on insect circulatory physiology.

***Gene expression.*** Given the potential impact of air pollution on olfactory communication (51), and the correlation with circulatory physiology shown in this study, we also assessed whether the observed differences in behavior, survival, and physiology also correlated to changes in gene expression in the antennae and heart of Giant Asian honey bees collected from the different sites ($n = 80$, 20 bees/site for antenna and heart, separately). Here, we concentrated on genes responsible for stress, metabolism, nutrition and defense, homeostasis and innate immunity, and cellular regulation as suggested from human health impacts. We performed comprehensive RNA sequencing (RNA-seq) analysis to assess the gene expression profiles from the antennae of all four sites. A total of 46, 29, and 39 differentially expressed genes (DEGs) were found expressed in bee antennae collected from low, moderate, and highly polluted sites when compared with rural site bees (Fig. 3*E* and *SI Appendix*, Fig. S2 *A–C*) using $P$ value $< 0.05$, and 1.5 log2FoldChange. We used UniProt (52) for gene ontology (GO) enrichment to identify the function and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (53) to define the pathways of the DEGs (Fig. 3*F* and *SI Appendix*, Table S3). A biomolecular interaction network was constructed using Cytoscape 3.6.1 (54) to visualize the relationships among genes. Gene ontology enrichment (53) of antennae-expressed genes revealed that all of the DEGs responsible for stress and lipid metabolism were up-regulated in bees collected in the highly polluted site when compared to rural bees (Fig. 3*F* and *SI Appendix*, Figs. S3*A* and S4 *A–C*). From gene ontology and pathway analysis of the heart transcriptome, we found all 14 identified genes responsible for stress and 60% of lipid metabolism-related genes were differentially expressed in bees collected from the highly polluted site when compared to bees from the low polluted site (Fig. 3*F* and *SI Appendix*, Figs. S3*B* and S4*E*).

We thus performed qRT-PCR using unexposed Giant Asian honey bees and bees collected from low and highly polluted sites ($n = 80$, 20 bees/site) with three biological replicates using nine differentially expressed genes (*SI Appendix*, Fig. S3), responsible for stress (vitellogenin-like, sterol-*o*-acyltransferase, and laccase-5-like), lipid metabolism (fatty acid synthase), nutrition and defense (major royal jelly protein), homeostasis and innate immunity (hymenoptaecin, cytochrome P450, and tyrosine 3-monooxygenase), and transcriptional regulation (histone H3). The KEGG pathway and gene ontology enrichment analysis showed that DEGs from antennal and heart tissue collected from *A. dorsata* at the different sites were involved in 10 different pathways (*SI Appendix*, Fig. S4). We selected specific genes from these pathways based on their biological significance for honey bees (such as known relationships with stress, metabolism, nutrition and defense, homeostasis, innate immunity, and cellular regulation) and common parameters like primer length, melting temperature (Tm), annealing temperature, $Mg^{2+}$ concentration, and other factors. The gene-specific primers used are listed in *SI Appendix*, Table S4.

Vitellogenin is a phospholipoglycoprotein (55) released from the fat body that circulates in the hemolymph (56). It protects cells from oxidative damage (57) and has incidentally been suggested to serve as a potential biomarker for neonicotinoid




| No. | Molecular/ biological functions | Number of genes upregulated in highly polluted condition | |
|---|---|---|---|
| | | Antennal transcriptome | Heart transcriptome |
| (1) | Stress response | 4/13 | 15/15 |
| (2) | Signaling pathway | 0 | 3/7 |
| (3) | Homeostasis and innate immunity | 2/9 | 6/7 |
| (4) | Lipid metabolism | 5/9 | 3/5 |
| (5) | Transcriptional regulation | 2/13 | 2/3 |
| (6) | Defense response | 1/1 | 0 |
| (7) | Homeostasis and glycogen metabolism | 8/8 | 0 |
| (8) | Signal transduction | 4/4 | 7/7 |
| (9) | Cuticle development | 2/2 | 0 |
| | Upregulated in highly polluted condition/ DEGs genes | 28/60 | 36/44 |

**Fig. 3.** Physiology, immunology, circulatory, and gene expression studies in the Giant Asian honey bee. (*A*) Respiration rate in µL $CO_2$/s [$n$ = 40/site; Welch's F (4, 93.74) = 15.28, $P < 0.0001$, Cohen's d (L vs. H) = 0.0004]. (*B*) Phenoloxidase (PO) activity measured at optical density at 490 nm [$n$ = 20/site; Welch's F (3, 40.05) = 2.34, $P = 0.087$, Cohen's d (L vs. H) = 0.062]. (*C*) Average SD (SD) between adjacent heartbeat intervals [$n$ = 30 segments averaged per 15 bees/site; Welch's F (3, 26.89) = 9.9, $P < 0.0001$, Cohen's d (L vs. H) = 1.81]. (*D*) THC in 10 µL of bee hemolymph [$n$ = 20 bees/site; Welch's F (4, 42.13) = 67.8, $P < 0.0001$, Cohen's d (L vs. H) = 1.61]. (*E*) Venn diagram of antennae transcriptome illustrating significantly differentially expressed genes from rural vs. low, moderate, and highly polluted conditions. (*F*) Number of genes with associated pathway up-regulated in bees collected at the highly polluted site vs. total number of differentially expressed genes (DEGs) for that pathway (39). (*G*) Results of the antennae qRT-PCR analysis for selected DEGs ($n$ = 20 bees/site; vitellogenin: U vs. L, t = 3.01, $P = 0.039$; U vs. H, t = 45.05, $P < 0.0001$; L vs. H t = 5.5, $P = 0.005$; sterol-*o*-acetyl transferase: U vs. L, t = 10.61, $P = 0.0004$; U vs. H, t = 109.8, $P < 0.0001$; L vs. H, t = 25.39, $P < 0.0001$; laccase-5: U vs. L, t = 13.55, $P = 0.0002$; U vs. H, t = 9.43, $P = 0.0007$; L vs. H, t = 16.5, $P < 0.0001$, two-tailed $t$ test). (*H*) Results of the heart qRT-PCR analysis for selected DEGs ($n$ = 20 bees/site; cytochrome P450: U vs. L, t = 3.86, $P = 0.018$; U vs. H, t = 21.62, $P < 0.0001$; L vs. H, t = 21.62, $P < 0.0001$; tyrosine 3-monooxygenase: U vs. L, t = 0.63, $P = 0.55$; U vs. H, t = 4.18, $P = 0.013$; L vs. H, t = 3.11, $P = 0.035$, two-tailed $t$ test). (*G* and *H*) number next to gene indicates pathway listed in *F*. Series with different letters denote significant differences (*A–D*, Welch ANOVA test followed by Dunnett's T3 multiple comparisons test and *G, H*, two-tailed $t$ test). See *SI Appendix*, Tables S5 and S6 for multiple comparison statistics. U = unexposed, R = rural, L = low, M = moderate, and H = highly polluted site. Scatter dot plots with error bars represent mean ± SD.

pesticide exposure in bees (58). Variation in worker behavior and lifespan is also correlated with vitellogenin depletion (59). Likewise, we observed low survival rates for Giant Asian honey bees from the highly polluted site (Fig. 1*E*) accompanied by low expression of vitellogenin in the antenna (Fig. 3*G*, two-tailed $t$ test). The sterol-*o*-acyltransferase gene is an evolutionarily conserved metabolic gene (60) that plays an important role in cellular cholesterol metabolism (61). In our study, the sterol-*o*-acyltransferase gene exhibited significantly increased expression in bees from the highly polluted site (Fig. 3*G*, two-tailed $t$ test). Laccases are important in cuticle sclerotization and toxin oxidation in insects (62), and cytochrome P450 monooxygenase is an important enzyme involved in detoxification and has also been implicated in bees' response to toxins such as pesticides (63, 64). These two genes correspondingly displayed increased levels of expression for antennal and heart tissue, respectively, for bees

collected from the highly polluted site (Fig. 3 *G* and *H*, two-tailed *t* test). Finally, the expression of tyrosine 3-monooxygenase, which is responsible for homeostasis and innate immunity, was significantly increased in heart tissue from bees collected from the highly polluted (Fig. 3*H*, two-tailed *t* test). Tyrosine 3-monooxygenase plays a major role in cuticle tanning, pigmentation, wound healing, and melanization of microbes and parasites during immune responses (49, 65). Interestingly, we did not see a correlation between the observed increase in tyrosine 3-monooxygenase expression and differences in phenoloxidase activity in bees from different sites, which also leads to melanin production for immune response (Fig. 3*B*). Perhaps this is because the change in tyrosine 3-monooxygenase activity is due to its other roles, or our physiological assay was not sensitive enough to detect the changes observed.

***Exposure of laboratory-reared and age-matched* Drosophila.** Our results indicate that Giant Asian honey bees exposed to RSPM exhibit significant differences in flower visitation behavior, heart rate, hemocyte levels, and ultimately survival. These differences are reflected by significant differences in the expression of genes related to stress, lipid metabolism, homeostasis, and innate immunity, which are also impacted in human health studies on air pollution. While the behavioral and physiological differences observed did not generally correlate with other potential factors such as date of collection, humidity, temperature, or wind speed, we did note a significant correlation of several of the measured parameters with the NDVI (Normalized Difference Vegetative Index; *SI Appendix*, Table S6, Pearson's correlation analysis). As a consequence, while we collected bees while foraging at nectar-rich *T. stans* at each site, it is possible that the bees were impacted by other differences in floral abundance, age, diet, source colony, or physiological condition between sites that we could not control in this field-based study. We note, however, that the NDVI index was only significantly different at the rural site (*SI Appendix*, Fig. S1*C*), implying that pollution, rather than vegetative availability, is a more probable factor driving the observed behavioral, physiological, and molecular differences in bees collected at the different sites.

Furthermore, the observed increases in RSPM and several toxic metals on the exoskeleton and internal tissues of field-collected bees using SEM-EDX suggest a direct relationship between exposure to RSPM and impacts on insect health and survival. Thus, to control for age, diet, source, and physiological differences between insects, we evaluated the survival, RSPM deposition, hemocyte levels, heart rate, and gene expression of laboratory-reared and age matched *Canton Special D. melanogaster* to laboratory conditions (C = control) as well as our low (L) and highly (H) polluted sites (66). Newly emerged flies ($n$ = 21,000, 7,000 flies/site, 1,000 flies per trial) were exposed for 10 d under similar shade and wind-protected locations and were provided with fresh food and water on alternate days. Under these conditions, flies exposed to the low and highly polluted sites showed significantly lower survival rates after 10 d of exposure (Fig. 4*A*; $n$ = 21 cages, 7 cages per site, 1,000 flies per cage, Welch ANOVA test followed by Dunnett's T3 multiple comparisons test). We then correlated the deposition of RSPM on the outermost layer for hindlegs, antennae, and wings with scanning electron microscopy (SEM) as performed for the Giant Asian honey bee (Fig. 4 *B*–*E*; $n$ = 450, 150/site), and after allowing flies to groom (67). We observed a similar pattern to that found for bees with significantly increased deposition of RSPM present on the edges and upper surface of the wings, the antennal tip, and on the hindlegs of flies exposed to the highly polluted site (Fig. 4 *C*–*E*; $n$ = 450 sections, 50 samples/section/site, Welch ANOVA test followed by Dunnett's T3 multiple comparisons test). We used SEM-EDX to determine the elements present on flies ($n$ = 150, 50 flies/site), which showed the presence of iron (Fe), manganese (Mn), silicon (Si), aluminum (Al), zinc (Zn), and copper (Cu) only on flies exposed to the highly polluted sites. However, the mean SD of interbeat interval in fly heart rate (Fig. 4*F*; $n$ = 60, 20 flies/site, Welch ANOVA test followed by Dunnett's T3 multiple comparisons test) and total fly hemocyte count (THC; Fig. 4*G*; $n$ = 2,400 flies, 20 trials/site, 40 flies/trial, Welch ANOVA test followed by Dunnett's T3 multiple comparisons test) were significantly different for laboratory-maintained flies, while the bee results also showed intersite differences. This discrepancy could be due to increased sensitivity in flies to pollution eliciting changes even at low pollution levels, the difference in length of exposure time to air pollution [10 d for flies vs. at least 22+ d for foraging bees (68)], or that the intersite effects in bees resulted from additional factors beyond air pollution.

We also performed qRT-PCR to compare the results obtained from bee experiments to laboratory-maintained flies and those exposed to our high and low polluted sites. We quantified the same genes in *Drosophila* we selected for bees for stress (yolk protein-1, sterol-*o*-acyltransferase, and laccase-2), lipid metabolism (fatty acid synthase), defense (yellow protein), homeostasis and innate immunity (cytochrome P450 and tyrosine 3-monooxygenase), and transcriptional regulation (histone H3-3a; Fig. 4*H*; $n$ = 180, 60 flies/site). Similar to our bee qRT-PCR, we found increased expression of cytochrome P450, sterol-*o*-acyltransferase, and tyrosine 3-monooxygenase in flies exposed to the highly polluted site (Fig. 4*H*, two-tailed *t* test). However, fly yolk protein displayed a significantly increased level of expression in flies from the highly polluted condition, unlike the analogous vitellogenin gene in bees. Interestingly, a previous study (69) showed that increased expression levels of *Drosophila* yolk protein family genes were negatively correlated with life span, opposite to vitellogenin for bees (59). Thus, these results do correspond to species-specific differences between stress-related genes and longevity between bees and flies.

Taken together, our results for both field-collected pollinators and laboratory-reared fruit flies indicate that RSPM is the most parsimonious cause for our observed molecular, cellular, physiological, morphological, and behavioral differences. In the case of the Giant Asian honey bee, *A. dorsata*, these effects could be either from direct or indirect exposure through contaminated food, water (70), or other substrates. These observed effects should now be subjected to controlled laboratory exposure studies to determine the specific RSPM components and levels at which these effects are observed. Surprisingly, increased RSPM levels did not correlate with quantitative changes in respiration rate or innate immunity, two physiological correlates most implicated for impacts of pollution on human health. Thus, increased air pollution may have unique and unexpected impacts on animal systems not anticipated from human studies alone. Nevertheless, our study offers an initial quantitative examination of the impacts of air pollution on insects, and shows that these molecular and physiological effects can be generalized to multiple insect orders (in this case, Hymenoptera and Diptera). Furthermore, our indication of molecular and physiological impacts on a migratory ecosystem service provider, the Giant Asian honey bee, suggest that the effects of urban air pollution could persist far from their point of origin.

Finally, the associated impacts of RSPM were observed at a $PM_{10}$ of roughly 50 $\mu g/m^3$ (our moderately polluted site). This level is equivalent to the "Interim Target-2" levels recommended by the World Health Organization for policy adoption (71). At these mean levels, we observed over 80% reduction in pollinator survival as well as significant molecular and physiological changes. We thus emphasize the urgency for more studies on wild plant and animal systems to better inform international air quality guidelines. Such studies are imperative to reveal the full impact of air pollution not only on human and environmental ecosystem health but, as in the case of this study, economic loss

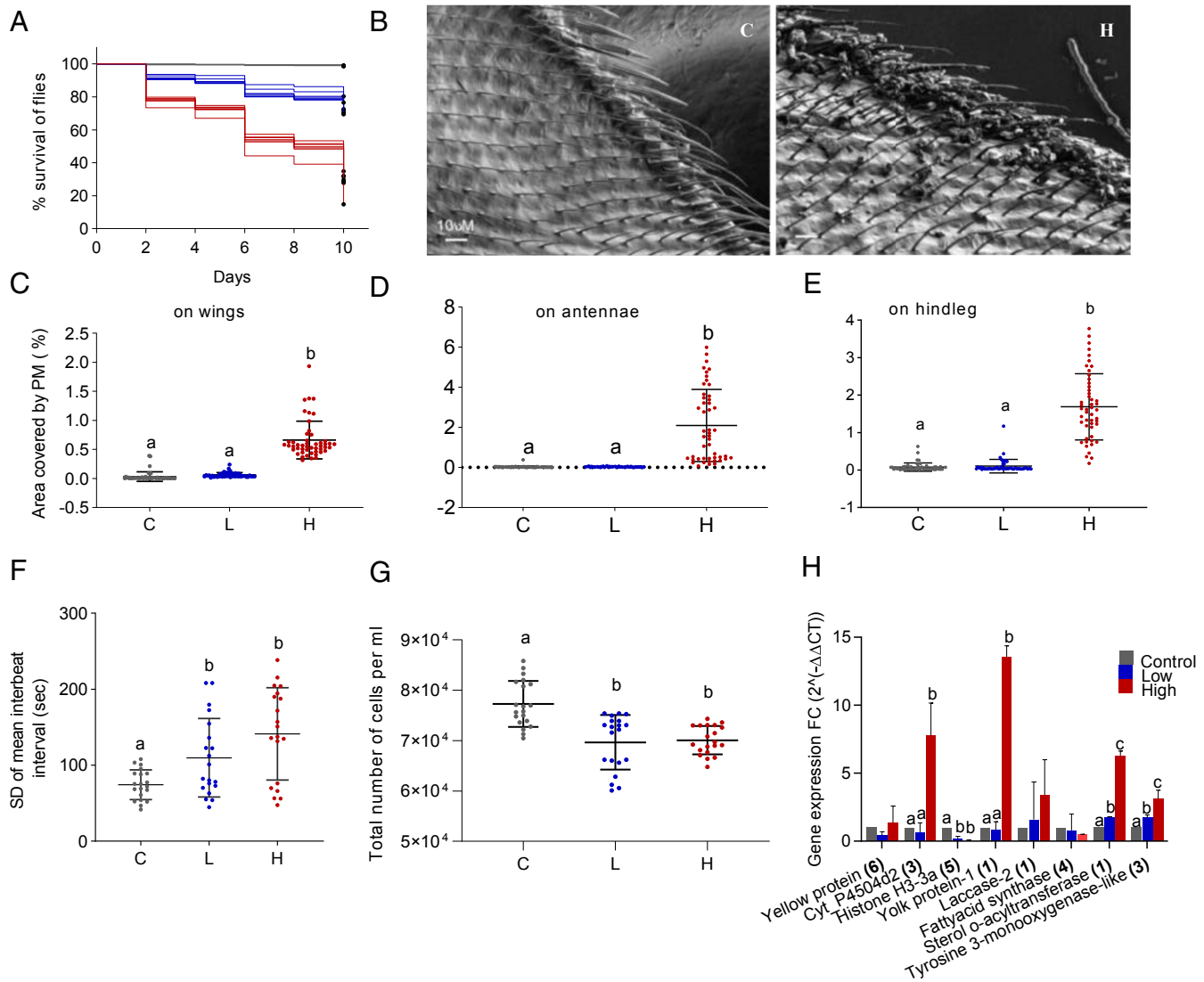



**Fig. 4.** Survival, morphology, circulatory, and gene expression studies in laboratory-reared and field-exposed *D. melanogaster*. (*A*) Kaplan–Meier survival curves with Log-rank (Mantel–Cox) test for percent survival of 10-d-old *Canton Special D. melanogaster* under field and laboratory conditions for 10 d ($n = 7$ cages of 1,000 flies each/site, $\chi^2 = 8{,}820$, df = 20, $P < 0.0001$). (*B*) Representative SEM images of wing bearing RSPM from control, C, and highly, H, polluted sites (2,000× magnification). (Scale bar: 10 μm). Percentage area covered by RSPM on wing [$n = 50$ samples/section/site (*C*); Welch's F (2, 77.63) = 86.23, $P < 0.0001$, Cohen's d (L vs. H) = 2.6], antennae (*D*) [Welch's F (2, 71.08) = 31.77, $P < 0.0001$, Cohen's d (L vs. H) = 1.61], and hindleg (*E*) [Welch's F (2, 82.73) = 80.34, $P < 0.0001$, Cohen's d (L vs. H) = 2.47]. (*F*) Average SD (SD) between adjacent heartbeat intervals [$n = 8$ segments averaged per 20 bees/site, Welch's F (2, 30.46) = 13.56, $P < 0.0001$, Cohen's d (L vs. H) = 0.56]. (*G*) THC in *D. melanogaster* hemolymph [$n = 20$ trials/site, 40 flies/trial, Welch's F (4, 42.13) = 67.8, $P < 0.0001$, Cohen's d (L vs. H) = 0.094]. (*H*) Results of *D. melanogaster* qRT-PCR analysis ($n = 180$, 60 flies/site), cytochrome P450: C vs. L, t = 0.85, $P = 0.44$; C vs. H, t = 4.9, $P = 0.008$; L vs. H, t = 4.9, $P = 0.007$; histone H3-3a: C vs. L, t = 8.29, $P = 0.0037$; C vs. H, t = 36.62, $P < 0.0001$; L vs. H, t = 1.23, $P = 0.03$; Yolk protein-1: C vs. L, t = 1.05, $P = 0.35$; C vs. H, t = 27.56, $P < 0.0001$; L vs. H t = 24.26, $P < 0.0001$; sterol-*o*-acetyltransferase: C vs. L, t = 21.84, C vs. H, t = 27.28, L vs. H, t = 23.1, $P < 0.0001$ and tyrosine 3-monooxygenase: C vs. L, t = 6.63, $P = 0.002$; C vs. H, t = 5.57, $P = 0.005$; L vs. H, t = 3.47, $P = 0.025$. Number next to gene indicates pathway listed in Fig. 3*F*. (*C*–*G*) Welch ANOVA test followed by Dunnett's T3 multiple comparisons test and *H*, two-tailed *t* test. See *SI Appendix*, Tables S5 and S6 for multiple comparison statistics. Series with different letters denote significant differences ($P < 0.05$). C = control, laboratory-maintained flies, L = low polluted site, H = highly polluted site. Scatter dot plots with error bars represent mean ± SD.

to pollinator-dependent crops and food security in highly polluted and vulnerable regions such as India.

## Materials and Methods

This study was performed to correlate the effect of air pollution on pollinator survival, floral visitation behavior, circulatory physiology, and gene expression. We used several approaches to assess Giant Asian honey bees, *A. dorsata*, from four sites selected with similar conditions except for varying air pollution levels: 1) field behavior to assess floral visitation and survival, 2) SEM-EDX to assess the particulate matter and its composition on the Giant Asian honey bee body, 3) respirometry, cardiac physiology, and biochemistry to assess impacts on respiratory, circulatory, and immune systems, respectively, 4) antennae and heart transcriptomics and qRT-PCR analysis to assess impacts on gene expression. We separately used these same approaches to measure survival, circulatory physiology, and gene expression in laboratory-reared and age-matched *D. melanogaster* exposed to laboratory conditions and our low and highly polluted sites as described in ref. 66 for 10 d with food and water. Sample numbers, data assembly, measurements, and statistical analyses are described in *SI Appendix, Materials and Methods*.

**Data Availability.** All data used in this study are made available within this article and *SI Appendix*.

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