Supplementary information for

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

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Field sites and insects tested

Bangalore is the third most populated city in India and located in the southeastern region on the state on the Deccan Plateau (Fig. 1A). The study was conducted from July 2016 to April 2019 in four localities of Bangalore. The study areas were selected (Table S1) according to the level of pollution namely: 1) Highly polluted: Peenya is biggest industrial area in the north of Bangalore city; 2) Moderately polluted: Lalbagh is a botanical garden in the center of Bangalore; 3) Low polluted: the campus of NCBS-TIFR (National Centre for Biological Sciences) situated on the north side of Bangalore; 4) Rural: ICTS (International Centre for Theoretical Sciences) on the rural northern outskirts of Bangalore.

The field experiments were conducted under natural conditions on Tecoma stans, a flowering ornamental plant found in all four sites. Particulate matter levels in all four sites were measured at PM$_{10}$ (particles less than 10 µM) using a portable pollution monitoring device (Smart Air Quality Monitor with Laser Sensor, Airveda, India) from 2017-2019, at the time of each bee collection or observation. Likewise, temperature, humidity and wind speed were measured using a portable weather station (WatchDog 2000 Series, Spectrum Technologies, Inc. Aurora, Illinois, USA). Giant Asian honey bees, A. dorsata were observed or collected randomly from T. stans while foraging at T. stans flowers. Multiple colonies were observed at each site, and A. dorsata is known to move its colonies throughout the year, so source colony could not be determined for individuals (http://bit.ly/beepollution). Foraging bees were observed or collected from T. stans flowers between 8 to 10 am from multiple sites simultaneously over several weeks for each type of analysis at common time periods to account for potential seasonal effects (Table S2). This time of day also encompassed the maximum foraging period for bees on this species (1). For a small number of analyses, unexposed bees were used to provide a control for pollution exposure in this species. These consisted of 3-5 day old bees obtained from a section of an A. dorsata colony with brood cells that was maintained in the incubator at 33°C. These bees had not been exposed upon emergence to environmental conditions outside of the incubator and were therefore considered “unexposed” as adults. Unfortunately, bees under these conditions did not survive to forager stage and therefore could not be age matched to the foragers (although we also could not decisively identify the age of the collected foraging bees either). Nevertheless, this was the only way we could present a true control for pollution exposure in this wild population.

In addition to direct weather parameters, the Normalized Difference Vegetative Index (NDVI) was calculated for 3.5 km around each site as a proxy for floral abundance. The chosen land area encompasses the maximum foraging range of this species from the sites of origin (2, 3). The response amplitude of NDVI value ranges from -1 to 1. Values between 0.2 to 0.4 represent the presence of vegetation in a specific area (4⇓-6). In this study, Sentinel-2 satellite data was used for mapping by separating and classifying the four different sites from the other land areas. The NDVI satellite images were used for vegetative classification by using ArcGIS software. Images were selected during each month of sampling as listed in Table S2.
We used wild type Canton Special strain of D. melanogaster adult flies maintained by the Drosophila Fly Facility at NCBS-TIFR for our lab-reared assays. Approximately 1000 newly emerged adult flies were placed in W47.5 x H93.0 cm² cages with fresh fly media and water changed on alternative days. We placed one cage in a 23°C insect rearing chamber at 60-75% RH and a 14 L-10 D cycle. Two other cages were placed at the low and highly-polluted sites close to the T. stans observation sites. Each cage was kept for 10 days. We repeated this exposure at each site 7 times (70 total exposure days, 10 days/trial). All cages were sheltered from the wind and direct sunlight. After 10 days of exposure, flies were collected and used for the various laboratory studies described.

**Field behavior and survival analyses**

Field observations were conducted from July 2016 to April 2017 as follows: 20 randomly selected T. stans inflorescences were observed for 5 min/inflorescence and all visitations by Giant Asian honey bee (A. dorsata) foragers were recorded for the presence of the pollinator inside the campanulate corolla of the individual flowers. This experiment was repeated on 20 days for each site at the same time period, observing multiple sites per week (Table S2). For survival analysis, 5 foraging bees were collected at a time over 10-15 visits to each site (Table S2) and were kept in a cage with 10% honey water ad libitum. Bees were observed at each hour and dead bees were removed from the cage. Bees that refused to eat or drink survived less than 4 hours and were not considered further in the analysis. The number of bees dying in the first 4 hours amounted to 9 (rural site, 14.8%), 5 (low, 8.6%), 11 (moderate, 16.9%), and 13 (highly polluted site, 18.1%) of the total bees collected, and their removal did not impact the total survival curves. The first 50 remaining bees were used for analysis. For Drosophila, the number of flies alive after 10 days was recorded for each of the 7 exposures. Dead flies were counted every 2 days while replacing food and water and removed throughout the test period.

For nectar measurement, inflorescences bagged (to avoid pollinator visitation) the evening before sampling (N = 240, 60/site) were measured from the T. stans plant between 8-10 am to coincide with pollinator visitation using 10µL graduated micro-capillary tubes. Nectar was drawn into the micro-capillary tubes by means of capillary action. The volume of withdrawn nectar/flower was then determined by measuring the rise in the volume of nectar within the tube (7).

**Morphometric, SEM and EDAX analysis**

We assessed the body weight and size of Giant Asian honey bees by measuring the wing area, and antennae and hindleg length and width using a dissection microscope (Olympus SZX10) with calibrated ocular micrometer for 120 bees from each site including unexposed bees. Images of antennae, wings, and hindleg of bees were taken under a dissection microscope. All measurements were performed with the use of ImageJ software (8).

Scanning electron microscopy and Energy Dispersive X-Ray Analysis (EDX) measurements were carried out using 2 wings, 2 antennae, and 2 hindlegs from each bee collected from the four sites. Samples were air-dried at room temperature [SEM; N = 80 bees, 20/site; 5 images/body part; 2 each of the flagellum (antenna), femur and tibia (hindleg) and edges of the forewing; 30 images/bee, 600 total images/site, EDX; N = 80, 20 bees/site]. No coatings or treatments were applied. Samples were scanned
between 1 to 2 kV to suppress the charging artifacts using a SEM MERLIN Compact VP equipped with EDAX. Similar to (9), we used ten different images of each body part for each bee at the same magnification (antennae, 300X, 450X, 600X, 720X, 800X; hindleg, 150X, 450X, 500X, 600X, 900X; wing, 100X, 250X, 350X, 550X, 650X). For each image, areas of PM coverage for that image were identified manually as regions of interest and the % area covered by PM calculated using ImageJ software (8). The energy dispersive X-ray (EDX) spectroscopy provides rapid analysis of the presence of elements on the honeybee body with minimum sample preparation time. However, due to technical limitations of the instrument and its accompanying software, only a qualitative analysis (presence/absence) of elements could be performed. EDAX analyses were assimilated in swapping sequence at the unchanged condition of 20kV. The same procedure was followed for SEM and EDAX experiment on flies (N = 450, 150/site, 50 samples/section).

**Transcriptomics and qRT-PCR**

The antennae and heart tissue of 20 Giant Asian honey bees per site (10 each for biological and technical replicate were dissected from bees randomly collected while foraging at *T. stans*, over 10-15 days total for all 4 sites in August, 2017, and February, 2018 respectively. Tissue was hand-dissected, transferred to RNAlater, stored at 4°C for one week, homogenized with a hand mortar, and finally pooled for further analysis. For heart transcriptome, 20 heart samples from bees collected at each site were dissected and stored in -80°C before sending for analysis (*Fig. S4D*). Total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to manufacturer’s guidelines. The concentration and quality of total RNA was examined using a Nanodrop Spectrophotometer and Qubit. Paired end cDNA library preparation for samples was performed by Genotypic Technology Pvt. Ltd., Bangalore, India (antennae samples), and BGI Genomics (heart samples). A reference-based transcriptome was performed for Giant Asian honey bee antennal samples. From 198.59 million Illumina HiSeq (150x2) reads 186.89 million high quality reads were processed of which ~74.37% of reads aligned to the reference genome of *A. dorsata* (https://www.ncbi.nlm.nih.gov/genome/?term=Apis%20dorsata). On average, 66.71% transcripts had a detectable FPKM value (>1) from the total expressed transcripts across all samples.

The raw data generated was checked for quality using FastQC and pre-processed by removing the adapter sequences and low quality bases. Preprocessing of the data was performed with Cutadapt2. The Tuxedo Suite protocol of TopHat (version2) was used to align the high quality data to the reference genome of *A. dorsata*. Cufflinks 2.2.1 was used to estimate and calculate transcript abundance. This results in normalized read count in the form of FPKM values. FPKM is a unit of measuring gene/transcript expression (Fragments Per Kilobase of transcript per Million mapped reads). Cuffdiff (10) was used to calculate the differentially expressed transcripts and categorize them into Up, Down and Neutrally regulated genes based on log2fold change values. All the identified genes were annotated by homology search against APIS data from UniProt (11), HymenopteraMine database (www.hymenopteragenome.org) and pathways were extracted from KEGG database (12). Common genes were identified between pathways and ontologies, and a network was constructed using Cytoscape 3.6.1 (13).
We used NCBI primer blast for designing the oligonucleotide primers as in (14) and ordered them from Sigma-Aldrich. The qRT-PCR assays were performed using KAPA SYBR® FAST qPCR master mix in Real-Time PCR Thermal cycler CFX96 (Bio-Rad, Hercules, CA). Three technical replicates were performed for each biological replicate in 30µl reactions; 15µl KAPA SYBR Green Supermix (Bio-Rad Laboratories), 0.75µM forward and reverse primer, and 3µl of 1:10 diluted cDNA sample (> 500ng/µl in all the samples). The cycling conditions were 95 °C for 5 sec, 39 cycles of 95 °C for 0.25 sec and 58 °C for 35 sec, followed by an annealing phase at 72 °C for 45 sec, with a melt curve analysis from 65 °C to 95 °C in 0.5 °C increases at 5 seconds per step. The relative expression levels of the target genes were normalized to the relative expression level of elongation factor 1 alpha (internal control) using the \(2^{\Delta\Delta Ct}\) method (15). For analysis, the relative expression of genes of each site was normalized against unexposed bees. A one-way ANOVA followed by Tukey HSD was used to interpret the expression differences between unexposed bees to low and highly polluted bees. The elongation factor 1 gene was used as internal control.

qRT-PCR analysis of flies was also performed using KAPA SYBR® FAST qPCR master mix in Real-Time PCR Thermal cycler CFX96 (Bio-Rad, Hercules, CA). The cycling conditions were 95 °C for 3 min, 39 cycles of 95 °C for 0.30 sec and 55 °C for 20 sec, followed by an annealing phase at 72 °C for 1 min, with a melt curve analysis from 65 °C to 95 °C in 0.5 °C increases at 5 seconds per step. Three technical replicates were performed for each biological replicate. The relative expression levels of the target genes were normalized to the relative expression level of elongation factor 1 alpha (internal control) using the \(2^{\Delta\Delta Ct}\) method (15). For analysis, the relative expression of genes from high and low were normalized against control flies. One-way ANOVA followed by Tukey HSD was used to interpret the expression differences between control to low and highly polluted flies. The RP49 gene was used as an internal control.

**Respirometry**

Respiration rates were measured from 20 Giant Asian honey bees from each site including newly eclosed-unexposed bees from a campus colony based on a modification of the protocol described in (16). The CO\(_2\) production rate was quantified for bees in a customized assay tube consisting of a 5 ml pipette tip attached to the capillary tube (10cm-length, 1.5mm-OD) and tightly sealed with wax. The pipette tip was then loaded with a small piece of sponge foam and filled with soda lime (Sigma-Aldrich) to around 1/4 volume of the tip. In each group, there were five replicates containing an individual bee/pipette with one respirometer without a bee used as a control for changes in atmospheric temperature and pressure. Bees were anesthetized on ice for few minutes. After releasing bees, we sealed the respirometer tightly to isolate it from external air temperature and pressure variation and allowed bees to recover from anesthetization for 5 min. An initial image of the chamber was taken to observe the level of liquid in each capillary tube against a scale bar. Bees were left in the chamber for 60 min, at which time a final image was obtained of the chamber including the scale bar. Images were processed with Fiji (8). By using the scale bar, we measured the change in liquid level and calculated the amount of produced carbon dioxide (µl/sec/bee).

**Phenoloxidase (PO) assays**
Hemolymph (~20µl) was extracted via syringe from twenty Giant Asian honey bees from each site in 500 µl of ice-cold phosphate buffered saline as described in (17). Extracted hemolymph with PBS buffer was frozen at -20°C at this point and thawed when all the samples were ready to be measured. The PO activity in the defrosted sample was assayed spectrophotometrically (Spectramax multimode reader) using L-dopa as a substrate (18). This involved pipetting triplicate 100 µl samples of the buffered hemolymph into a microtiter plate, adding 100 µl of 20 mM L-dopa to each and incubating the mixture at 25°C for 30min. The absorbance was read at 490 nm (UV/VIS SP-80001).

Heart rate analysis

Giant Asian honey bees were collected from each field condition (N = 60, 15/site) and kept in a cage with sugar water solution and pollen for more than an hour before starting recording. After the specified time period (1 hour) the bees were anaesthetized on ice for 2 min and the abdomen carefully dissected. As can be seen in SI Appendix, Movie S1 (note particularly the highly polluted condition), the bee heart did not always exhibit uniform beating along its length. To account for this, the heart system was divided into 30 equal segments from anterior to posterior (Fig. S4D), and randomized videos of the heartbeat were then obtained for 5 minutes each with 10 sections at a time. (Movie S1). After recording, all the videos were converted into an image sequence and processed using ImageJ software (8). First, we calculated the average number of beats per minute (BPM) for each segment. We then measured the interbeat interval (IBI) by calculating the mean time interval between heartbeats per segment. Studies suggest IBI is a more accurate and reliable metric than BPM (19, 20). In all 30 sections, the overall average pixel intensity of a region of interest outlining the heart sections was used for measuring BPM and IBI. By using a custom-built Python script, the average standard deviation (21) (SD) between adjacent heartbeat intervals from bees of different sites was calculated and compared. The same procedure was used for all the bees and flies from different field sites. As the size of the fly heart is smaller compared to bees, we divided the fly heart into 8 segments and then used the same described protocol for measurement.

Hemocytology

Total hemocyte count (THC) (22) was observed for individual Giant Asian honey bees from each site (Fig. L4) using a hemocytometer (Marienfeld, Germany) (N = 20/site). Hemolymph samples were removed using a 200 µl pipette from the dissected abdomen of the bee. Hemocyte identification was performed as in (22). Total hemocyte counts (THC) were measured by transferring 20 µl of hemolymph diluted with 10 µl of 1x phosphate buffer, pH 7.2 to a Neubauer hemocytometer. THC was expressed as the number of hemocytes/ml of hemolymph. THC of flies (23) from low, high and control flies were observed using hemocytometer as mentioned above (N = 2400, 800/site). Here, we pooled 40 flies in each replication to get 1 µl of hemolymph. Hemocyte identification was performed as in (24).

Statistical Considerations

We hypothesized that populations of Giant Asian honey bees collected from sites with varying levels of pollution would exhibit differences in ethology, morphology, toxicology, respirometry, circulatory physiology, immunology and gene expression.
Collected data for each type of analysis was first assessed for normality using a D'Agostino-Pearson normality test computing skewness and kurtosis (25). We then tested for equal variance using a Brown-Forsythe test. While the datasets did not deviate from normality, some data did reflect heteroscedasticity. We thus compared all population means using a Welch analysis of variance (Welch ANOVA) followed by Dunnett’s T3 test at P-value < 0.05. Both of these tests do not assume equal variance between populations. Median survival times were estimated using Kaplan-Meier survival analysis and differences in the time distribution between groups were carried out for statistical significance using Log-rank (Mantel-Cox) test for bees and flies. For qRT-PCR, two-tailed t-tests were performed between quantified gene expression from unexposed (U) bees or control (C) flies and other groups.

As noted in the main text, while the observed significant differences between honey bee populations did not correlate with other potential factors such as temperature, humidity, season or obvious local environmental and ecological differences (Fig. S1, Table S1), we could not control for differences in bee age, diet, source colony, or physiological condition between sites due to the wild and unmanaged nature of this bee in Bangalore. These factors could increase variability in the data. We thus accounted for this limitation in two manners. As a direct measure, we evaluated survival, RSPM deposition, hemocyte levels, heart rate, and gene expression in lab-reared and age matched Canton Special D. melanogaster exposed to lab conditions (C = control) as well as our low (L) and highly (H) polluted sites (26) over a period of 10 days, repeated 7 times. This allowed us to control age, diet, source, and physiological differences in an alternate insect system and assess if our observed effects in the honeybee were reproducible in controlled populations (see Field sites and insects tested in Supplementary Methods).

Secondly, while increased sampling can generally overcome variability in the data due to sampling effects and confounding variables, we were limited to 455 sampled bees per site due to mortality and risk of oversampling colonies and inducing colony absconding in the middle of sampling. As a consequence, we assessed statistical confidence in our data by measuring effect size, which indicates the magnitude of the phenomenon. Cohen’s d (27), an effect size index, was calculated as the difference between two means divided by a standard deviation for the data. Generally, values of 0.2 are considered a small effect, 0.5 medium, and 0.8 large. Cohen’s w was alternatively calculated for contingency data (used for the survival analysis) as the degree of difference between the distribution specified by the alternate hypothesis and the null hypothesis (which in this case was equal mortality at 50%). Generally, values of 0.2 are considered a small effect, 0.3 medium, and 0.5 large. Comparing populations from our low vs. highly polluted sites produced values ranging up to 8.32 showing that our tested populations exhibited large differences. These effect sizes also indicate that the collected sample sizes were sufficient to indicate differences in the population and overcome variability.

We further composed a correlation matrix to find correlations between the variables which displayed significant differences. Linear correlations were considered by calculating a Pearson's Correlation Coefficient (r), which is a linear correlation coefficient that ranges between -1 and +1; -1 for a strong negative correlation, +1 for a strong positive correlation and 0 for no correlation. Table S6 reports the Pearson correlation matrix for the dependent and independent variables.
We report Welch’s F or the Chi-square statistic and degrees of freedom, T-test t, the associated P value, and Cohen’s d or w assessed for populations collected from the low (L) vs. highly (H) polluted sites with each figure panel where significance has been found. Results of all Dunnett’s T3 comparisons are reported in Table S5. All figures and statistics were obtained using Graphpad prism software 8, GraphPad Software. Inc, California, USA and SPSS Statistics software 26.
Fig. S1. Local environmental and ecological parameters and morphometric measurements in the Giant Asian honey bee. (A) Plot of average monthly measurements of PM$_{10}$ µg/m$^3$ for each study site during 2016-2019. (B) Monthly mean temperature (Welch’s F (3, 137.3) = 1.26, p = 0.28), Monthly mean humidity (Welch’s F (3, 136.6) = 2.27, p = 0.08) and monthly mean wind (Welch’s F (3, 135.9) = 18.42, p <0.0001). See Table S2 for dates of measurement, , W = Winter, S = Summer, M = Monsoon season. (C) Vegetative cover as NDVI for 3.5 km radius around each site from 2017 to 2018 encompassing the honey bee experiments, Welch’s F (3, 48.66) = 57.2, p < 0.0001 (D) Average number of observed flowers at each site per inflorescence (Welch’s F (3, 38.66) = 149.9, p <0.0001, Cohen’s d =5.32). (E) Nectar measurements from _T. stans/_flower (Welch’s F (3, 131) = 2.07, p = 0.106). Morphometric measurements in the Giant Asian honey bee in mm (N = 20/site) for (F) Hind legs (Welch’s F (3, 39.49) = 2.46, p = 0.076), (G) Antennae (Welch’s F (3, 41.8) = 2.01, p = 0.12) Wings (Welch’s F (3, 41.68) = 1.73, p = 0.17) and (H) Wings (Welch’s F (3, 41.68) = 1.73, p = 0.17). Antennae (Welch’s F (3, 41.8) = 2.01, p = 0.12). (I) Intertegular distance of bees in µm (Welch’s F (3, 40.46) = 1.71, p = 0.17). (J) Average beats per minute (BPM) (N = 15 bees/site; Welch’s F (3, 30.28) = 0.543, p = 0.65). (K) Total weight of bees in mg (N = 30/site, Welch’s F (4, 71.4) = 2.11, p = 0.08). (L) Representation of Giant Asian honey bee wing assessed for wing damage. Note the absence of wing tear on edge of wing. Series with different letters denote significant differences (Welch ANOVA test followed by Dunnett’s T3 multiple comparisons test). U = Unexposed, R = Rural, L = Low, M = Moderate and H = Highly polluted condition, W = Winter, S = Summer, M = Monsoon. Error bars represent mean ± SD.
**Fig. S2. Volcano Plots.** Volcano plots depicting the fold difference of gene expression in Giant Asian honey bee antennae collected from (A) low (B) moderately and (C) highly polluted sites when compared with bees from the rural site. Red dots represent significantly differentially expressed genes with log2 fold ≥ 1.5.
Fig. S3. Site-specific gene expression. (A) Heat map of site-specific gene expression in Giant Asian honey bee antennae collected from rural vs. low, moderately, and highly polluted sites, respectively. (B) Heat map of site-specific gene expression for Giant Asian honey bee heart tissue genes collected from low vs. highly polluted sites. Numbers below each section indicate pathway listed in Fig. 3F and Fig. S4.
Fig. S4. GO enrichment analysis of differentially expressed genes in Giant Asian honey bee antennae and heart. Pathways represented by differentially expressed genes for bee antennae collected from (A) Low (B) Moderately and (C) Highly polluted sites when compared with the rural site. Pathways are divided into modules represented by different colors and corresponding to pathways listed in Fig. 3F. (D) The bee dorsal vessel after removal of gut contents and stinger. (E) GO enrichment analysis as of differentially expressed genes in the heart tissue of bees collected from the highly polluted site when compared with the low polluted site. Size of node represents the number of enriched genes differentially expressed in each set. Network generated using Cytoscape (12).
## Table S1. Field site selection criteria

| Variables                              | Rural       | Low         | Moderate | High       |
|----------------------------------------|-------------|-------------|----------|------------|
| Shade from other trees                 | No          | No          | No       | No         |
| Distance from road                     | 150 m       | 120 m       | 140 m    | 170 m      |
| Traffic on nearest road                | Very low    | Very low    | Very High| Medium     |
| Evidence of Plant Disease              | No          | No          | No       | No         |
| Species of Tecoma                      | T. stans    | T. stans    | T. stans | T. stans   |
| Pesticide application                  | No          | No          | No       | No         |
| Average Temperature in °C (2016-2019)  | 26.99       | 26.63       | 27.28    | 27.03      |
| Average Humidity in % (2016-2019)      | 51.93       | 49.36       | 51.23    | 51.02      |
| Average wind speed in km/h (2016-2019) | 2.753       | 3.207       | 2.716    | 2.818      |
| Nearby human activities                | Research    | Research    | Tourism  | Industries |
| Latitude N                             | 13°08′46.80″ | 13°04′17.51″ | 12°57′02.85″ | 13°01′44.82″ |
| Longitude E                            | 77°30′51.86″ | 77°34′45.80″ | 77°35′05.21″ | 77°31′22.81″ |
| Presence of Giant Asian honey colonies | Yes         | Yes         | Yes      | Yes        |

*a* Data obtained from portable weather station *WatchDog 2000 Series.*

*b* Locations of colonies can be found at: [http://bit.ly/beepollution](http://bit.ly/beepollution).
### Table S2. Calendar of work plan (2016-2019)

| Experiments performed                                | 2016 | 2017 | 2018 | 2019 |
|------------------------------------------------------|------|------|------|------|
| Field site and animal selection                      |      |      |      |      |
| Field behaviour studies                              |      |      |      |      |
| PM10 measurements                                    |      |      |      |      |
| Temperature, humidity and wind measurement           |      |      |      |      |
| Heart rate measurements – honey bees                 |      |      |      |      |
| Total blood cell count – honey bees                  |      |      |      |      |
| SEM and EDAX, morphometry - honey bees               |      |      |      |      |
| Respiration rate – honey bees                        |      |      |      |      |
| PO activity – honey bees                             |      |      |      |      |
| Survival rate - honey bees                           |      |      |      |      |
| Transcriptome and qRT-PCR (antennae)                 |      |      |      |      |
| Transcriptome and qRT-PCR (heart)                    |      |      |      |      |
| Survival rate - Drosophila                           |      |      |      |      |
| SEM and EDAX - Drosophila                            |      |      |      |      |
| Total blood cell count - Drosophila                  |      |      |      |      |
| qRT-PCR - Drosophila                                 |      |      |      |      |
| Nectar measurements - Tecoma stans                   |      |      |      |      |
### Table S3. Differentially expressed genes (DEG) from antennae and heart transcriptome of the Giant Asian honey bee with respective Fragments Per Kilobase Million (FPKM) values along with their function.

| Gene names                                                                 | FPKM Value_1 | FPKM Value_2 | Functions                  |
|----------------------------------------------------------------------------|---------------|--------------|----------------------------|
| Chitinase-like_protein_idg64-like                                         | 0.69647       | 0.30535      | Cuticle development         |
| Keratin_type_ii_cytoskeletal_1-like                                       | 0.727171      | 0.272829     | Cuticle development         |
| Acyl-coa_dela(11)_desaturase-like                                         | 0.727213      | 0.272787     | Homeostasis and glycogen metabolism |
| Dihydroxypropylene-residue_succinyltransferase_component_of_2-oxoglutarate_dehydrogenase_complex_mitochondrial-like | 0.67952       | 0.32048      | Homeostasis and glycogen metabolism |
| E3 ubiquitin-protein_ligase_bowel-1-like                                  | 0.667815      | 0.32185      | Homeostasis and glycogen metabolism |
| Organic_cation_transporter_protein-like                                   | 0.726954      | 0.273046     | Homeostasis and glycogen metabolism |
| Phosphatidylinositol_transfer_protein_alpha_isomer-like                   | 0.676248      | 0.332752     | Homeostasis and glycogen metabolism |
| Phosphoglycerate_kinase-like                                             | 0.703807      | 0.296913     | Homeostasis and glycogen metabolism |
| Protein_diauxide-isomerase_a3-like                                       | 0.674556      | 0.325444     | Homeostasis and glycogen metabolism |
| Protein_diauxide-isomerase-like                                          | 0.688358      | 0.311642     | Homeostasis and glycogen metabolism |
| Ryanoctine_receptor_448-like                                             | 0.67623       | 0.32377      | Homeostasis and glycogen metabolism |
| Titin-like                                                                | 0.746613      | 0.253387     | Homeostasis and glycogen metabolism |
| Ubiquitin-4-like                                                         | 0.727694      | 0.272306     | Homeostasis and glycogen metabolism |
| Zinc_finger_MIZ_domain-containing_protein_1-like                         | 0.773121      | 0.226879     | Homeostasis and glycogen metabolism |
| Alpha-tocopherol_transfer_protein-like                                    | 0.523968      | 0.676032     | Homeostasis and innate immunity |
| Annexin-6b-like                                                          | 0.31487       | 0.68513      | Homeostasis and innate immunity |
| Aplidinun_type_13-like                                                   | 0.795945      | 0.204055     | Homeostasis and innate immunity |
| Catalase-like                                                            | 0.686933      | 0.313067     | Homeostasis and innate immunity |
| Cytochrome_F450_6A1-like                                                 | 0.331538      | 0.668462     | Homeostasis and innate immunity |
| Hymenoptaecin-like                                                      | 0.954254      | 0.045476     | Homeostasis and innate immunity |
| Metalloproteinase_inhibitor_3-like                                       | 0.325193      | 0.674807     | Homeostasis and innate immunity |
| Transferrin-like                                                         | 0.744511      | 0.255489     | Homeostasis and innate immunity |
| Tyrosine_3-monoxygenase-like                                             | 0.835842      | 0.164158     | Homeostasis and innate immunity |
| Angiotensin-converting_enzyme-like                                       | 0.516548      | 0.683452     | Lipid metabolism            |
| Antithrombin-III-like                                                   | 0.68233       | 0.31767      | Lipid metabolism            |
| Elongation_of_very_long_chain_fatty_acids_protein-like                   | 0.32431       | 0.67569      | Lipid metabolism            |
| Esterase_E4-like                                                        | 0.788934      | 0.211066     | Lipid metabolism            |
| Insitol_monophosphatase_3-like                                           | 0.322404      | 0.677596     | Lipid metabolism            |
| Major_royal_jelly_protein_5-like                                         | 0.270481      | 0.729519     | Lipid metabolism            |
| Platelet_glycoprotein_V-like                                             | 0.813305      | 0.180699     | Lipid metabolism            |
| Putative_fatty_acyl-coa_reductase_CG5065-like                            | 0.306971      | 0.699029     | Lipid metabolism            |
| Actin-binding_Rho_activating_protein-like                                | 0.732058      | 0.267942     | Stress response             |
| Aldose_reductase-like                                                   | 0.711625      | 0.288375     | Stress response             |
| Battenin-like                                                           | 0.67964       | 0.32036      | Stress response             |
| Cuatomer_subunit_alpha-like                                             | 0.702495      | 0.297505     | Stress response             |
| Guanine_nucleotide-binding_protein_G(i)_subunit_alpha-like               | 0.315979      | 0.684021     | Stress response             |
| Ion_transport_peptide-like                                              | 0.293113      | 0.706887     | Stress response             |
| Lacase-5-like                                                           | 0.225355      | 0.774645     | Stress response             |
| MAP_kinase-interacting_serine/threonine-protein_kinase_2-like           | 0.68638       | 0.31362      | Stress response             |
| Neurotirnin-like                                                        | 0.732356      | 0.267644     | Stress response             |
| Pecanex-like_protein_1-like                                             | 0.738818      | 0.261182     | Stress response             |
| Plectrstrin_homology_domain-containing_family_M_member_1-like            | 0.676048      | 0.323952     | Stress response             |
| Protein_sel-1_homolog_1-like                                            | 0.701529      | 0.298471     | Stress response             |
| Putative_inorganic_phosphate_cotransporter-like                         | 0.327162      | 0.672838     | Stress response             |
| Serine/threonine-protein_phosphatase_PPI1-beta-like                     | 0.685594      | 0.314406     | Stress response             |
| Splicing_factor_arginine/serine-rich_15-like                             | 0.712525      | 0.287475     | Stress response             |
| Sterol_O-acyltransferase_1-like                                         | 0.32746       | 0.67254     | Stress response             |
| Vitellogenin-like                                                       | 0.17271       | 0.82729      | Stress response             |
| 60_kda_heat_shock_protein_mitochondrial-like                            | 0.687219      | 0.312781     | Transcriptional regulation  |
| Actin_cloze_205-like                                                    | 0.691597      | 0.308403     | Transcriptional regulation  |
| Biorientation_of_chromosomes_in_cell_division_protein_1-like            | 0.510198      | 0.689802     | Transcriptional regulation  |
| Cyclin-dependent_kinase_5-like                                          | 0.207638      | 0.792262     | Transcriptional regulation  |
| Importin_subunit_alpha_7-like                                           | 0.714825      | 0.283573     | Transcriptional regulation  |
| L-lactate_dehydrogenase-like                                            | 0.744176      | 0.255824     | Transcriptional regulation  |
| Multidrug_resistance_protein_homolog_49-like                            | 0.700666      | 0.299334     | Transcriptional regulation  |
| NF-kappa_B_inhibitor_cactus-like                                       | 0.68616       | 0.31384      | Transcriptional regulation  |
| Nuclear_receptor_coactivator_3-like                                     | 0.711662      | 0.283338     | Transcriptional regulation  |
| Protein_TAP1_homolog                                                   | 0.723039      | 0.279691     | Transcriptional regulation  |
| Gene Name                                           | FPKM Rural | FPKM High | Function                        |
|----------------------------------------------------|------------|-----------|---------------------------------|
| Retinal-specific ATP-binding cassette transporter-like | 0.705437   | 0.294563  | Transcriptional regulation      |
| T-complex protein 1 subunit delta-like              | 0.688213   | 0.311787  | Transcriptional regulation      |
| V-type proton ATPase 116 kDa subunit A isoform 1-like | 0.694501   | 0.303499  | Transcriptional regulation      |
| **Heart transcriptome DEG**                         |            |           |                                 |
| Serine/threonine-protein kinase 32B-like            | 24.2862    | 10.7589   | Stress response                 |
| Protein phosphatase PHLPP-like protein-like         | 97.0074    | 40.2084   | Signaling pathway               |
| Cytochrome P450 4c3-like                           | 45.2052    | 17.6482   | Homeostasis and Innate immunity |
| Irregular chiasm C-roughest protein-like           | 22.153     | 9.63891   | identical protein binding       |
| Fatty acid synthase-like                           | 145.053    | 70.5307   | Lipid metabolism                |
| Elongation of very long chain fatty acids protein 1-like | 3461.52  | 1297.88   | Lipid metabolism                |
| DNA damage-regulated autophagy modulator protein 2-like | 52.1507  | 19.7999   | regulation of autophagy         |
| Lysine-rich repeat-containing protein 70-like       | 30.3197    | 11.5906   | Signaling pathway               |
| Forkhead box protein P2-like                       | 11.5283    | 5.53716   | transcription                   |
| Organic cation transporter protein-like             | 27.1158    | 10.3408   | Signaling pathway               |
| Alpha-tocopherol transfer protein-like              | 18.3544    | 89.1166   | Homeostasis and Innate immunity |
| Cytochrome P450 9e2-like                           | 33.5445    | 98.7185   | Homeostasis and Innate immunity |
| Cytochrome P450 6A1-like                           | 214.854    | 445.94    | Homeostasis and Innate immunity |
| Cytochrome P450 6A1-like                           | 228.425    | 531.113   | Homeostasis and Innate immunity |
| Melittin-like                                      | 214.27     | 6218.2    | Homeostasis and Innate immunity |
| Tyrosine 3-monoxygenase-like                       | 5.14111    | 10.6032   | Homeostasis and Innate immunity |
| Peritrophin-1-like                                 | 35.693     | 125.407   | Lipid metabolism                |
| Phospholipase A2-like                              | 8.87424    | 376.733   | Lipid metabolism                |
| Cuticle protein 16.9, isoform B-like                | 321.635    | 655.393   | Lipid metabolism                |
| Histone H3-like                                    | 15.3942    | 32.2258   | Signaling pathway               |
| Full gene name – *Apis dorsata* | Sequence (5'->3') | Size (bp) | Tm (℃) | GC (%) |
|-------------------------------|-------------------|-----------|---------|--------|
| Cytochrome P450 9e2-like (LOC102673102) FP | GTACGTGGGGGATGTACGAGTTCA | 23 | 61.98 | 52.17 |
| Cytochrome P450 9e2-like (LOC102673102) RP | TAATCCGGGGAATCTTTCCGCA | 23 | 63.88 | 47.83 |
| Fatty acid synthase-like (LOC102675377) KP | ATGCTACGAATCGCTTTCA | 20 | 57.51 | 45 |
| Fatty acid synthase-like (LOC102675377) RP | ACCATCGATATTGTGTTTAG | 20 | 50.38 | 35 |
| Tyrosine 3-monoxygenase-like (LOC102675778) FP | ACGGTGCAAAAAACTGCGCT | 20 | 64.44 | 55 |
| Tyrosine 3-monoxygenase-like (LOC102675778) RP | ACGTACGATCGGAGCCCTCT | 20 | 64.01 | 65 |
| Histone H3-like (LOC102674139) KP | CTAATAAGCTCTGTAAGAGTG | 21 | 56.48 | 47.62 |
| Histone H3-like (LOC102674139) RP | TTCTTGAAGAGCCATGACAGC | 21 | 58.57 | 47.62 |
| Fatty acid synthase-like (LOC102675377) FP | ATGCAACCTTTTACCCCTACA | 23 | 59.44 | 43.48 |
| Fatty acid synthase-like (LOC102675377) RP | ACCATCGATATTGTGTTTAG | 20 | 50.38 | 43.48 |

| Full gene name – *Drosophila melanogaster* | Sequence (5'->3') | Size (bp) | Tm (℃) | GC (%) |
|-------------------------------------------|-------------------|-----------|---------|--------|
| yellow-d-FP | TCGACGATTTCCACCATGAGCGT | 23 | 71.6 | 47.8 |
| yellow-d-RP | CGTACGGGTGTCTTTGCTCTT | 22 | 68.6 | 54.5 |
| cytochrome P450 4d2 -FP | CGACAAGCGGGTGTTACAATGG | 21 | 67.4 | 52.3 |
| cytochrome P450 4d2 -RP | GTGCGTCGACGCTCTTTGCTTT | 22 | 76.5 | 63.6 |
| histone H3.3A-FP | CAGCTGCAATGATGATGACCT | 21 | 58.4 | 42.8 |
| histone H3.3A-RP | CAGCTGCAATGATGATGACCT | 20 | 70.2 | 54.5 |
| yolk protein 1-FP | GCCAAGCCCACTGCTTCATT | 20 | 71.9 | 60 |
| yolk protein 1-RP | GCCAAGCCCACTGCTTCATT | 21 | 68.3 | 47.6 |
| Laccase 2-FP | CATGTGGCCACTCTTACACATTTAAG | 24 | 59.8 | 55 |
| Laccase 2-RP | CATGTGGCCACTCTTACACATTTAAG | 20 | 66.6 | 55 |
| FASN1 - Fatty acid synthase 1-FP | CCAACATGCTGACACCCATC | 21 | 69.6 | 52.3 |
| FASN1 - Fatty acid synthase 1-RP | CTGAACATGTGACACCCATC | 19 | 70.2 | 57.8 |
| Tyrosine 3-monoxygenase-FP | CATGCTGCAATGATGATGACCT | 24 | 69 | 41.6 |
| Tyrosine 3-monoxygenase-RP | TGTGCTGCAATGATGATGACCT | 23 | 67 | 43.7 |
| Sterol o-acyltransferase-FP | AAGCTGCAATGATGATGACCT | 23 | 68.3 | 47.8 |
| Sterol o-acyltransferase-RP | TTGCATATTGCGAAAAAGACCTCG | 24 | 71.3 | 45.8 |
| RP49 (CG7939)-FP | CGATATGCAAGCTAAAGCA | 20 | 62.8 | 45 |
| RP49 (CG7939)-RP | GGGCGATCTACGACAGTAT | 20 | 64.2 | 55 |
Table S5. Summary of Dunnett’s T3 post hoc statistics. See Figures 1 – 4 and S1 for details.

| Fig. | U vs R | U/C vs L | U vs M | U/C vs H | R vs L | R vs M | R vs H | L vs M | L vs H | M vs H |
|------|--------|---------|-------|---------|-------|-------|-------|-------|-------|-------|
|      | t | p | t | p | t | p | t | p | t | p |
| 1C   | 3.437 | 0.0044 | 7.516 | <0.0001 | 12.9 | <0.0001 | 5.39 | <0.0001 | 11.97 | <0.0001 | 9.127 | <0.0001 |
| 1D   | 6.551 | <0.0001 | 8.669 | <0.0001 | 12.6 | <0.0001 | 3.53 | 0.0081 | 10.52 | <0.0001 | 9.178 | <0.0001 |
| 2J   | 3.232 | 0.0251 | 0.283 | 0.9998 | 8 | <0.0001 | 11.61 | <0.0001 | 10.35 | <0.0001 | 8.612 | <0.0001 |
| 2K   | 0.675 | 0.9827 | 1.405 | 0.653 | 12.8 | <0.0001 | 1.772 | 0.3996 | 12.85 | <0.0001 | 12.4 | <0.0001 |
| 2L   | 8.008 | <0.0001 | 2.628 | 0.0754 | 14.6 | <0.0001 | 7.795 | <0.0001 | 16.88 | <0.0001 | 15.55 | <0.0001 |
| 3A   | 3.2 | 0.02 | 4.89 | <0.0001 | 5.2 | <0.0001 | 5.8 | <0.0001 | 2.284 | 0.2215 | 3.257 | 0.0189 | 2.62 | 0.0995 | 1.389 | 0.8301 | 0.023 | >0.9999 | 1.499 | 0.7589 |
| 3B   | 2.48 | 0.104 | 0.07 | >0.99 | 2.65 | 0.06 | 1.86 | 0.34 | 0.12 | >0.9999 | 1.885 | 0.331 |
| 3C   | 2.776 | 0.0632 | 0.56 | 0.99 | 3.7 | 0.0096 | 1.583 | 0.535 | 4.966 | 0.001 | 3.86 | 0.0057 |
| 3D   | 0.79 | 1 | 6.24 | <0.0001 | 2.4 | 0.1896 | 12 | <0.0001 | 5.256 | <0.0001 | 1.551 | 0.7216 | 10.4 | <0.0001 | 3.729 | 0.0063 | 5.106 | 0.0003 | 8.905 | <0.0001 |
| 4C   | 2.15 | 0.0996 | 13 | <0.0001 | 12.87 | <0.0001 |
| 4D   | 0.05 | >0.999 | 8 | <0.0001 | 8.008 | <0.0001 |
| 4E   | 0.82 | 0.7989 | 13 | <0.0001 | 12.38 | <0.0001 |
| 4F   | 2.87 | 0.0247 | 4.7 | 0.0003 | 1.772 | 0.2296 |
| 4G   | 4.79 | <0.0001 | 6 | <0.0001 | 0.299 | 0.9867 |

S1B/T  2.447 | 0.093 | 0.275 | 0.9998 | 0.33 | 0.99 | 2.384 | 0.1079 | 1.505 | 0.5765 | 0.503 | 0.9966 |

S1B/H  1.343 | 0.6947 | 0.802 | 0.9619 | 1.14 | 0.8245 | 1.963 | 0.2733 | 0.162 | >0.9999 | 1.777 | 0.3838 |

S1C  8.95 | <0.0001 | 9.76 | <0.0001 | 10.19 | <0.0001 | 1.4 | 0.653 | 1.88 | 0.347 | 0.22 | >0.99 |

S1D  12.09 | <0.0001 | 11.84 | <0.0001 | 2.42 | 0.1215 | 1.311 | 0.7174 | 16.84 | <0.0001 | 15.36 | <0.0001 |

S1E  2.274 | 0.1385 | 0.372 | 0.994 | 0.51 | 0.9962 | 1.993 | 0.2556 | 1.793 | 0.3719 | 0.157 | >0.9999 |

S1F  1.396 | 0.6591 | 2.551 | 0.0872 | 0.56 | 0.9338 | 0.59 | 0.9913 | 0.967 | 0.9078 | 2.004 | 0.27 |

S1G  0.816 | 0.9568 | 2.111 | 0.22 | 2 | 0.2704 | 1.38 | 0.6967 | 1.33 | 0.704 | 0.156 | >0.9999 |

S1H  1.164 | 0.8107 | 2.119 | 0.2157 | 0.31 | 0.9998 | 1.031 | 0.8808 | 0.717 | 0.9765 | 1.556 | 0.5456 |

S1J  0.329 | 0.9997 | 0.531 | 0.995 | 1.94 | 0.3056 | 0.311 | 0.9997 | 1.75 | 0.4137 | 0.761 | 0.9678 |

S1K  0.0823 | 0.8 | >0.9999 | 0.3557 | 0.9995 | 1.06 | 0.865 | 0.3213 | 0.9997 | 1.154 | 0.8152 | 0.608 | 0.9895 |

S1K  0.8 | 1 | 0.94 | 0.9836 | 1.4 | 0.8155 | 0.1 | >0.9999 | 2.285 | 0.2244 | 0.907 | 0.987 | 0.64 | 0.9992 | 2.513 | 0.1405 | 1.123 | 0.9463 | 1.295 | 0.8802 |
Table S6. Summary of correlation matrix.

| Days   | Humidity | Temperature | Wind | PM10 | Vegetation cover | No. of bees observed | Heart rate | SEM | THC |
|--------|----------|-------------|------|------|------------------|----------------------|------------|-----|-----|
| Days   | 1        |             |      |      |                  |                      |            |     |     |
| Humidity |         |             |      |      |                  |                      |            |     |     |
| Temperature |         |             |      |      |                  |                      |            |     |     |
| Wind   |          |             |      |      |                  |                      |            |     |     |
| PM10   |          |             |      |      |                  |                      |            |     |     |
| Vegetation cover |          |             |      |      |                  |                      |            |     |     |
| No. of bees observed |          |             |      |      |                  |                      |            |     |     |
| Heart rate |          |             |      |      |                  |                      |            |     |     |
| SEM    |          |             |      |      |                  |                      |            |     |     |
| THC    |          |             |      |      |                  |                      |            |     |     |

** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

Non-significant

c. Cannot be computed because at least one of the variables is constant.

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Movie S1. Sample heartbeat videos of Giant Asian honey bees collected from low and highly polluted sites.
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