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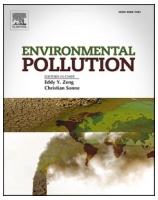

Repeated short-term exposure to diesel exhaust reduces honey bee colony fitness☆

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ABSTRACT

Production of insect-pollinated crops is often reliant on honey bee (*Apis mellifera*) pollination services. Colonies can be managed and moved to meet the demands of modern intensified monoculture farming systems. Increased colony mortalities have been observed, which are thought be caused by interacting factors including exposure to pesticides, parasites, viruses, agricultural intensification, and changes in global and regional climate. However, whilst common tropospheric air pollutants (e.g. NO_x, particulate matter etc) are known to cause a range of negative effects on human health, there is little evidence of their impact on the health of *A. mellifera*. This study investigates the effects of exposure to diesel exhaust on *A. mellifera*, both at the level of individual foragers and on the whole colony. We exposed a series of colonies to diesel exhaust fumes for 2 h a day over the course of three weeks and contrasted their performance to a series of paired control colonies located at the same field site. We investigated markers of neuronal health in the brains of individual foragers and measured the prevalence of common viruses. Electronic counters monitored daily colony activity patterns and pollen samples from returning foragers were analysed to investigate plant species richness and diversity. The amounts of honey, brood and pollen in each colony were measured regularly. We demonstrated an upregulation of the synapse protein Neurexin 1 in forager brains repeatedly exposed to diesel exhaust. Furthermore, we found that colonies exposed to diesel exhaust lost colony weight after the exposure period until the end of the summer season, whereas control colonies gained weight towards the end of the season. Further investigations are required, but we hypothesise that such effects on both individual foragers and whole colony fitness parameters could ultimately contribute to winter losses of honey bee colonies, particularly in the presence of additional stressors.

1. Introduction

Managing the balance between effective food production and protection of natural resources, including vital ecosystem services, is becoming more difficult, primarily due to increased resource demands from a growing global population (Alexandratos and Bruinsma, 2012; FAO, 2009). Animal pollination is an important ecosystem service for food production, with around 75% of food crop species depending on insect pollination (Klein et al., 2007).

Changes in land use and habitat specificity affects pollinator composition (Bommarco et al., 2012; Carré et al., 2009; Hallmann et al., 2017; Winfree et al., 2011) and a global decline in numbers of terrestrial insect species, including pollinator species has been identified (Carvalheiro et al., 2013; Klink et al., 2020; Potts et al., 2010; Powney et al., 2019). With respect to pollinating bee species, populations of wild bees are experiencing a sustained period of decline in both abundance and species richness (Biesmeijer et al., 2006; Potts et al., 2010; Powney et al., 2019). As a managed pollinator species honey bees are less dependent on landscape characteristics and can be moved to locations suitable for them or where they are required (Woodcock et al., 2013). Therefore, honey bee colonies can, to some extent, be used to mitigate the effects of wild pollinator losses for the pollination of certain food crops (Stern et al., 2001; Woodcock et al., 2013). However, in the last decade, beekeepers world-wide have also reported increased yearly losses of managed honey bee colonies (Currie et al., 2010; Ellis et al., 2010; Jacques et al., 2017); up to 40% in the USA (Kulhanek et al., 2017)

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and 29% in South-Africa (Pirk et al., 2014). A recent study reported average winter colony losses of 16% in 36 participating countries (including 33 European countries) with variation between countries ranging from 2% to 32.8% (Gray et al., 2019).

The evidence is that declines in both managed and wild pollinators are due to multiple interacting factors, with no single outright cause (van der Sluijs et al., 2013; Vanbergen and Initiative, 2013; vanEngelsdorp et al., 2009). Pesticides, agricultural intensification, pathogens, including viruses, and the consequences of climate change are implicated as stressors. Pollinator colonies exposed to a combination of simultaneously occurring stressors could be pushed over a critical tipping point and as a consequence the colony may collapse (Lever et al., 2014). With respect to honey bees, a colony may be capable of overcoming the challenge of each individual stressor, although it may be fatal to individual honey bees, but if pre- or simultaneously exposed to one or more other stressors, it may be rendered more vulnerable and therefore less able to endure the insult (Bryden et al., 2013; Reitmayer et al., 2019).

In this study, we investigate the effects of one such stressor – diesel exhaust – on different parameters of both individual forager honey bees and colony success. Air pollution is a significant issue with many deleterious implications for human health (Brunekreef and Holgate, 2002; Weinberger et al., 2001), yet its potential health effects on lower order animals, such as insects, has received limited investigation (Petters et al., 1983; Reitmayer et al., 2019; Thimmegowda et al., 2020) or has been focused on indirect effects by investigating the influence of air pollution on host-parasite relationships or ecosystem functions (Bell et al., 2011; Braun and Flückiger, 1985; Lee et al., 2012). Whilst, studies have demonstrated that nitrogen oxides (NO_x) from diesel exhaust can alter the composition of the floral odours that honey bees use to locate flowers, with potentially negative implications for honey bee's foraging and fitness (Girling et al., 2013; Lusebrink et al., 2015; Ryalls et al., 2022), there have been few investigations into the direct effects of air pollution on honey bee individuals or colonies (Leonard et al., 2019a; Leonard et al., 2019b; Reitmayer et al., 2019). However, one recent field study of Giant Asian honey bees, *Apis dorsata*, demonstrated significant correlations between increased particulate matter deposition and reductions in bee survival and health (Thimmegowda et al., 2020).

Diesel exhaust is a major contributor to urban air pollution, it is a complex mixture of organic and inorganic compounds, with both gaseous (e.g. NO_x and sulphur dioxide) and particulate matter (PM) (Schnaibel and Grieshaber, 2004; WHO, 2000). Road transportation contributes 40–70% of global NO_x emissions with diesel engines being the biggest contributor with 85% of all NO_x emission from transportation vehicles (Lee et al., 2013; Wang et al., 2012). In mammals, components of diesel exhaust have been shown to cause neuronal damage, neuro-inflammation and alter blood brain barrier functions (Gerlofs-Nijland et al., 2010; Hartz et al., 2008; Levesque et al., 2011).

We have previously shown that short-term exposure to diesel exhaust is detrimental to the learning abilities of forager honey bees (Reitmayer et al., 2019). Here we investigate whether repeated short-term exposure to diesel exhaust would alter cellular expression of proteins associated with learning and memory in the central nervous system (CNS) of forager honey bees. The functions of neurons and glial cells in the honey bee brain mirror those in mammalian brains (Brandt et al., 2005; Haehnlein and Bicker, 1997). Morphological changes in the CNS occur with changes in behaviour over the forager life span (Winnington et al., 1996). Draper protein is expressed in ensheathing glial cells in insects and is required for the clearance of degenerating neurons after injury (Doherty et al., 2009; MacDonald et al., 2006) and remodelling during development (Melcarne et al., 2019). Neurexins (NRX) are found in the pre-synaptic compartment and are essential to the development of the circuitry required for the establishment of memory formation (Chen et al., 2011; Dean and Dresbach, 2006; Südhof, 2008). Forager honey bees must find and memorise foraging sites; therefore, effective processing and memorising of new information is required for successful collection of nectar and pollen.

The wider aim of this study was to conduct a first investigation into whether repeated short-term exposure to diesel exhaust, functions as a stressor contributing to declines of honey bee colonies. To examine this in colonies repeatedly exposed to diesel exhaust and in paired control colonies, we investigated whether it was possible to detect changes in parameters relevant to both individual forager and whole colony fitness and success.

2. Methods

2.1. Honey bee colonies

All experimental animals were Buckfast bees (*Apis mellifera* hybrid). Treatment hives were generated by dividing hives. Two hives were equally split to each establish one control and one diesel exhaust treatment nucleus hive. A single larger hive was used to generate two diesel exhaust treatment and two control nucleus hives. Therefore, eight Langstroth nucleus hives, four for diesel exposure and four controls, were used for the experiments. Each nucleus had six frames and was equipped with a new mated German Buckfast queen (Becky's Bees, Andover, Hampshire, UK). Hive checks were carried out weekly (see Fig. S1) distilled water mist was sprayed onto the top frame of the hive, if needed, to avoid use of a conventional smoker during hive husbandry. Monitoring of *Varroa destructor* mites was performed via visual inspections of the frames because *Varroa* screens cannot be used in standard nucleus hives. During hive checks low numbers of *Varroa* mites (up to 2 mites per hive over the course of the experiment) were detected in all nucleus hives.

The experiment was conducted at a University of Southampton field site (+50° 57′ 49.77″, −1° 25′ 23.14″, Chilworth, Hampshire, United Kingdom, see Fig. S2). The four hives for each treatment (control and diesel exhaust exposure) were housed in their own tarpaulin enveloped cage (2 × 2 × 2 m), with the south-west face of the cage left open and all hive entrances facing in this direction. The hives were placed on wooden pallets to shield them from soil moisture and low ground temperatures (see Fig. 3). Counting units were attached to the entrances of each hive (see Fig. S3F and G) and were powered by 12V vehicle batteries (indicted by arrow in Fig. S3F). The colonies exposed to diesel exhaust were located near a diesel generator (SDE3000, Suntom, indicated by white asterisk in Fig. S3A), the control colonies were located a sufficient distance away (~60 m, upwind) to avoid contamination from diesel exhaust (see Fig. S2B, c: position of control colonies, d: position of diesel exhaust treated colonies). Therefore, the available foraging area and other environmental conditions did not vary between treatments (see Fig. S2).

2.2. Repeated short-term exposure to diesel exhaust

A silicon tube (4 m × 8 mm diameter, Thermo Fisher) was used to duct exhaust gases from the generator to the diesel exhaust treatment tarpaulin cage (see Fig. S3A and B); the silicon tubing was attached to the ceiling of the cage (see Fig. S3E, arrows indicating flow direction of diesel exhaust). The tube was attached to the exhaust pipe of the diesel generator using silicon tubing (50 cm × 3 cm diameter) and a glass connector (see Fig. S3C and D). The connections were not sealed off and only part of the exhaust created by the generator was routed to the diesel exhaust treatment cage. Gas pressure created by the generator was sufficient to move the generated exhaust into the tarpaulin cage. During exposure periods, both tarpaulin cages were closed at the front except for a 50 cm opening at the bottom to allow any remaining active foragers to return to the hive. The front side of the tarpaulin cages was left open at all other times. Exposures were carried out for 2h per day for a duration of 20 days. Exposures started 100 min before calendar sunset time for each day (see Fig. S1). To ensure that forager bees collected for analysis were only exposed to diesel exhaust as adults, exposures were

limited to a period of 20 days. Worker honey bees perform in-hive tasks for ca. 21 days before starting to forage. This ensured that no collected foragers had been exposed to diesel exhaust during their larval development. Control samples of foragers used for molecular analysis and pollen samples, as well as frame images were taken from all hives on the afternoon before the first exposure to diesel exhaust was conducted (control timepoint, see Fig. S1).

Gaseous components of diesel exhaust were measured in both the diesel exhaust treatment and control tarpaulin cages in a trial experiment, using a toxic gas probe (TG501+; Graywolf Sensing Solutions), at different time points (5, 10 and 30 min) after starting the generator. The probe was placed in the centre of the tarpaulin cage between the two central hives (Fig S3B). Levels of both oxygen and carbon monoxide remained constant between treatments and time points (see Table S1). In both diesel exhaust and control cages the concentrations of nitric oxide (NO), nitrogen dioxide (NO_2) and sulphur dioxide (SO_2) remained below the detection limits of the probe for all measurements. Therefore, even in the diesel exhaust exposed tarpaulin cages NO remained below 200 ppb, NO_2 remained below 20 ppb and SO_2 remained below 100 ppb. A preliminary study indicated that this generator produced NO and NO_2 in an approximate ratio of 0.6:1, which would suggest that levels of NO would have also been below the probes NO_2 detection limit of 20 ppb. These readings were comparable to ambient pollution levels in Southampton City Centre (ca. 7 km from the study site) during the course of the experimental period (Table S1). To put these values further into context, taking a mean of the average monthly recordings of NO, NO_2 and SO_2 in London, UK, between January 2010 and August 2019, provides concentrations of 59.6 ppb, 28.1 ppb and 1.2 ppb respectively for roadsides, and 16.9 ppb, 17.1 ppb and 1.2 ppb respectively for background measurements (King's College London, 2020).

2.3. Neuronal health and CNS function of individual foragers

2.3.1. Recording expression of neurexin 1 and draper

Expression levels of neurexin 1 (NRX1) and draper (drpr) were determined for brain samples of foragers. Bees were sampled from paired hives, two control and two diesel exhaust treated. Returning forager honey bees carrying a pollen load were collected at two different time points (10 bees per hive per time point, i.e., a total of 80 bees): the control timepoint (0d) and 10 days into the repeated short-term exposure (see Fig. S1).

To collect returning foragers the entrance gates of the hives were blocked with a plywood plate causing returning foragers to congregate at the landing platform. Individual foragers were trapped by placing a 50 ml Falcon tube over their body and immediately immobilized by cooling them on ice. Heads were removed and stored in RNAlater (Ambion) at −20 °C. The thorax and abdomen were frozen and stored at −20 °C.

The brain was dissected out of the head capsule, homogenized in Trizol® Reagent (Invitrogen) and RNA extracted according to manufacturer's instructions. DNase treatment was performed to eliminate genomic DNA (DNase 1, Invitrogen). RNA concentrations of the individual samples were measured using a NanoDrop Spectrophotometer (Thermo Fisher); 260/280 nm ratios were used to assess sample purity. Reverse transcription polymerase chain reaction was performed using the iScript™ Select cDNA Synthesis Kit (BioRad) containing Moloney Murine Leukemia Virus reverse transcriptase. Oligo (dt)primers were used for amplification.

Primers used to analyse the expression of NRX1(NM_001145740.1, fwd: TTCGGACCAGGAAAAGGAATC, rev: GTACAGCATCGTTTACGCTTG, 112bp) and drpr (XM_006559982.1, fwd: CGAGGCAAGAAACGTACACAG, rev: ACACTTACAGACATCGGGTG, 275bp) in honey bee brain tissue were designed using NCBI Primer-Blast. Primer sequences for ribosomal protein L8 (RPL8) were obtained from Collins et al. (2004). Primers were tested and optimal annealing temperature (30s, 55° C) was assessed using REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich) according to manufacturer's instructions. Quantitative PCR (qPCR) was carried out to measure expression levels of NRX1 and drpr; qPCR was performed using Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Fisher) according to manufacturer's instructions.

2.3.2. Analysis of gene expression data

Cycle threshold (Ct) values were obtained using the MJ Opticon Monitor Quantification Software (BioRad). Expression levels were normalized to Ct values of the housekeeping gene RPL8 and ΔCt values obtained from the control timepoint group of the corresponding hive. A univariate analysis of variance test was used to investigate the effects of treatment and parental hive and their interaction (SPSS v24).

2.4. Viral prevalence in hives

2.4.1. Honey bee sampling

Bees were sampled from paired hives, two control and two diesel exhaust treated. Bees were collected at three different time points (10 bees per hive per time point, i.e., a total of 120 bees): at the control time point, 10 days and 20 days into the exposure (see Fig. S1). Each bee was tested for a range of RNA viruses, Israeli acute paralysis virus (IAPV), Deformed Wing Virus (DWV) and Black Queen Cell Virus (BQCV).

2.4.2. PCR analysis of viral prevalence and viral load

RNA extraction of body samples was carried out as described above for brain samples. All primers used, were specific to bind to viral cDNA and are not compatible with honey bee cDNA, therefore no DNAse treatment was performed. Primer sequences were obtained from Hernán Sguazza (2013). Primers for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as a housekeeping gene, were designed using NCBI Primer-Blast (fwd: CGCTTTCTGCCCTTCAAATG, rev: CTTCGCAAATCTATTCACTCGG). The GAPDH primer pair is exon-exon junction spanning. The analysis was carried out using a multiplex PCR approach. Primers were tested individually, and conditions optimized for each set of primers before running the analysis as multiplex PCR.

To assess viral replication rates of DWV, qPCR was carried out with samples that showed a positive result in the virus screening. The qPCR reaction was performed using the Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Fisher) according to manufacturer's instructions.

2.4.3. Analysis of viral load

Cycle threshold values were obtained using MJ Opticon Monitor Quantification Software (BioRad). Expression levels were normalized against Ct values of GAPDH. Virus genome copy numbers were compared using a Kruskal-Wallis test (SPSS v24).

2.5. Hive activity

2.5.1. Honey bee counting units

Honey bee counting units were designed to count every occasion that an individual honey bee left or entered the hive, to provide a measure of hive activity. In brief, the hardware of the honey bee counting unit consisted of a counter board, a microcontroller board, sensors and a hive entrance adapter. The honey bee counting unit system used infrared reflection sensors for movement detection. A hive entrance adapter separated the hive entrance into individual gates through which the honey bees had to leave or enter the hive on a one-by-one basis (see Fig. S4 and associated text in the supplementary materials for details).

Hive activity was recorded during the 20-day exposure period as well as three days before and after the exposure period (see Fig. S1). Foraging data was recorded 24h a day, 7 days a week. Malfunction of the counting units caused by severe rainfall and hardware failure resulted in a reduced number of days with continuous foraging activity data. The counting units had to be modified after two days of activity to account for field conditions that were not previously encountered during pre-

testing. In addition, days on which hive manipulations took place were excluded because those manipulations have a strong influence on the counting data outcome. In total there were seven days of data suitable for further analysis.

2.5.2. Statistical analysis of hive activity data

To investigate whether treatment (diesel vs control) influenced total hive activity (separately for whole days, day-time and night-time [as dictated by sunrise and sunset each day]) over the entire experimental period (7 sampling days) the data was analysed using a series of factorial repeated measure ANCOVAs with treatment (diesel or control) as a between-subject factor, date as a within-subject factor and hive parentage (coded as dummy variables) as covariates. Hive parentage was not a significant factor in any of the models and was therefore removed from the models for the reported analyses. Shapiro-Wilk tests of normality indicated that distribution of the dependent variable in each combination of the related groups (date by treatment) was normal. The outputs of the ANCOVAs demonstrated that the data met assumptions of sphericity and homogeneity of variance, except for the night-time only data, which did not satisfy tests of homogeneity of variance and was therefore log transformed and subsequently found to meet all tests or normality, sphericity and homogeneity of variance.

To provide a measure of whether hive activity increased, decreased, or was constant during the treatment period, the mean total activity for each treatment over the course of the experiment was plotted, a linear line of best fit calculated and the slope of each line recorded. This was performed for activity during: i) whole days, ii) daytime only, and iii) night-time only. Slopes for each treatment were compared by a series of one-way ANOVAs with treatment and hive parentage as fixed factors, but with no interaction included in the model due to a lack of degrees of freedom. For each ANOVA hive parentage had no significant effect and was therefore removed from the model. All statistics were conducted using SPSS v24.

2.6. Pollen foraging

2.6.1. Pollen composition analysis

Pollen samples were collected from the hind legs of returning foragers to identify the plant species it derived from. Pollen samples were collected at the control time point, 10 and 20 days into the exposure period, and three weeks after the last exposure day (3wp, see Fig. S1). For each timepoint, pollen from 10 bees per hive was sampled from paired hives, three control and three diesel exhaust treated. Acetolysis of pollen samples was performed according to published protocols and Safranin-O staining was used to improve contrast prior to light microscopic analysis (Jones, 2014). Imaging was carried out using a Zeiss Axioplan 2 microscope equipped with MetaMorph imaging software. Four pictures per pollen sample were taken of random areas on the slide. Each picture was analysed for the pollen species present in the image, identified based on size and morphology of the grain. If more than one species were present, all pollen grains of each individual species were counted and the percentage of each species in relation to all pollen grains counted in all four pictures was calculated.

2.6.2. Analysis of pollen composition

A series of metrics from the pollen data for each treatment at the four different collection time points was assessed: i) mean species richness per hive; ii) mean number of novel species per hive since the previous recording; iii) Simpson's Diversity Index for each treatment at each time point; and iv) Sørensen coefficient of similarity for each time point. Because only three hives were sampled per treatment statistical analyses were not conducted on these data.

2.7. Hive product composition and weight over time

2.7.1. Measurement of honey, pollen and brood content

Photographic images from hive frames were used to assess the amounts of honey, pollen and brood in all hives. During each hive check, images of both sides of each frame were taken. Hives were opened and the honey bees on each frame were shaken off. Pictures were taken using a Sony Alpha A57 camera. Hive pictures were taken at the control time point, 10 and 20 days into the exposure and three, six and nine weeks after the end of the exposure (3wp, 6wp, 9wp, see Fig. S1). Pictures were number coded for later reassignment to the different treatment hives and analysis was performed blind and without bias.

Honey stores were estimated using a grid which was placed over the images. Frame pictures were divided into 12 segments which facilitated determination of the percentage of the frame covered with sealed honey. If squares were not completely filled with honey, an estimate was made of how many squares the honey filled areas would cover. Precision of this estimation technique was confirmed by comparing estimated honey filled areas with actual cell counts of ten randomly chosen frames; variation between methods was less than 3%. Area estimation was carried out by the same person for all frames. For brood and pollen counts each cell filled with either brood or pollen was counted individually; because cells filled with pollen and brood are usually more scattered across a frame.

2.7.2. Statistical analysis of honey, pollen and brood content

To investigate whether treatment (diesel vs control) influenced the frame contents (honey, brood and pollen) over the entire experimental period, data were analysed initially using a series of factorial repeated measures ANCOVAs with treatment (diesel or control) as a between-subject factor, date as a within-subject factor (excluding the first day of recording, as this was a control measure) and hive parentage (coded as dummy variables) as covariates (SPSS v24). Hive parentage was not a significant factor in any of the models and was therefore removed from the models for the reported analyses. For honey storage data, Shapiro-Wilk tests of normality indicated that distribution of the dependent variable in each combination of the related groups (date by treatment) was not normal and therefore these data were square-root transformed and retested to confirm this assumption was met. Both brood and pollen storage data were found to be normal. For honey (square-root transformed), brood and pollen (untransformed), the outputs of the ANCOVAs demonstrated that the data met assumptions of sphericity and homogeneity of variance.

2.7.3. Hive weight

To monitor weight development of hives over the course of the study, weight measurements were taken before the start of the exposure, at the end of the exposure period and three, six and nine weeks after the end of the exposure period (see Fig. S1). Straps were permanently attached to the hives in a balanced position, so that when lifted by the strap the hive remained level (see Fig S3F). To weigh the hives, the hook of a commercially available scale (designed to weigh luggage, and with a minimum graduation of 10 g) was looped under one of the hive straps, and the hive was then lifted by the handle on the scale and the weight recorded.

2.7.4. Statistical analysis of hive weight

A series of t-tests were used to investigate differences between changes in hive weights during the duration of the experiment (SPSS v24).

3. Results

3.1. Effect of diesel exhaust exposure on neuronal health

Analysis of the expression of the glial cell marker drpr 10 days after

the start of the exposure showed no effect of treatment ($F_{1,36} = 0.54$, P = 0.82) but a significant effect of parental hive ($F_{1,36} = 4.35$, P = 0.044) and no interaction between treatment and parental hive ($F_{1,36} = 0.16$, P = 0.69, Fig. 1A). The effect of parental hive was mainly driven by individuals from one parental hive showing a greater variation of drpr expression levels with two distinct groups (high and low) of drpr expression.

Analysis of the expression of the pre-synaptic transmembrane protein NRX1 gene showed a significant effect of treatment ($F_{1,36} = 19.48$, P < 0.001) but no effect of parental hive ($F_{1,36} = 0.40$, P = 0.53) and no interaction between treatment and parental hive ($F_{1,36} = 3.76$, P = 0.06, Fig. 1B). Individuals from diesel exhaust treatment hives had significantly elevated NRX1 gene expression levels.

3.2. Virus screening of hives

None of the tested animals were positive for IAPV. Mean infection rate for BQCV was 8.3% across both treatments, with no significant effect of date (Wald Chi-square = 0.0, P = 1.0) or treatment (Wald Chi-square = 0.0, P = 1.0) and no interactions between date and treatment (Wald Chi-square = 0.0, P = 1.0). Mean infection rate for DWV was 56.7% across both treatments. There was a significant effect of date (Wald Chi-square = 12.048, P = 0.002) but not of treatment (Wald Chi-square = 1.01, P = 0.31) and a significant interaction between date and treatment (Wald Chi-square = 7.94, P = 0.02, Fig. 1C).

3.3. Hive activity

The total mean daily activity of all hives over the course of the study was unimodal (Fig. 2A and B). There was a significant effect of date on mean daily hive activity ($F_{6,30} = 3.46$, P = 0.01; Fig. 2C), and whilst visually total activity appeared to be higher in the diesel exhaust treated hives, there was no statistically significant effect of treatment ($F_{1,5} = 2.60$, P = 0.17) and no interaction between date and treatment ($F_{6,30} = 0.96$, P = 0.47; Fig. 2A and B). For mean daytime activity only, there was a significant effect of date ($F_{6,30} = 2.83$, P = 0.03) but no effect of treatment ($F_{1,5} = 2.44$, P = 0.18) and no interaction between date and treatment ($F_{6,30} = 0.91$, P = 0.50). For mean night-time activity only, there was a significant effect of date ($F_{6,30} = 4.63$, P = 0.002) but no effect of treatment ($F_{1,5} = 4.6$, P = 0.09) and no interaction between date and treatment ($F_{6,30} = 0.46$, P = 0.83).

Over the course of the study, mean daily activity appeared to decrease for diesel exhaust treated hives and increase for control hives (Fig. 2C), which seemed to be driven by changes in daytime (Fig. 2D) rather than night-time (Fig. 2E) activity. However, there was significant variation in these data with large confidence intervals around the means for each treatment and no significant differences between the slopes of lines for diesel exposed and control hives either overall ($F_{1,6} = 0.17$, P = 0.70), during daytime only ($F_{1,6} = 0.17$, P = 0.69) or during night-time only ($F_{1,6} = 0.33$, P = 0.86).

3.4. Pollen load of returning foragers

In total 17 different pollen species (see Fig. S5 for representative images) were identified from the pollen load of returning foragers of all control and diesel exhaust treated hives at four different time points. Most foragers returned with their corbicula filled with pollen from only one plant species. If more than one plant species was present, a main plant species making up more than 80% of the total pollen could clearly be identified in all but one sample.

There were few differences in species richness between the two treatments over the four timepoints (see Table S2). However, hives

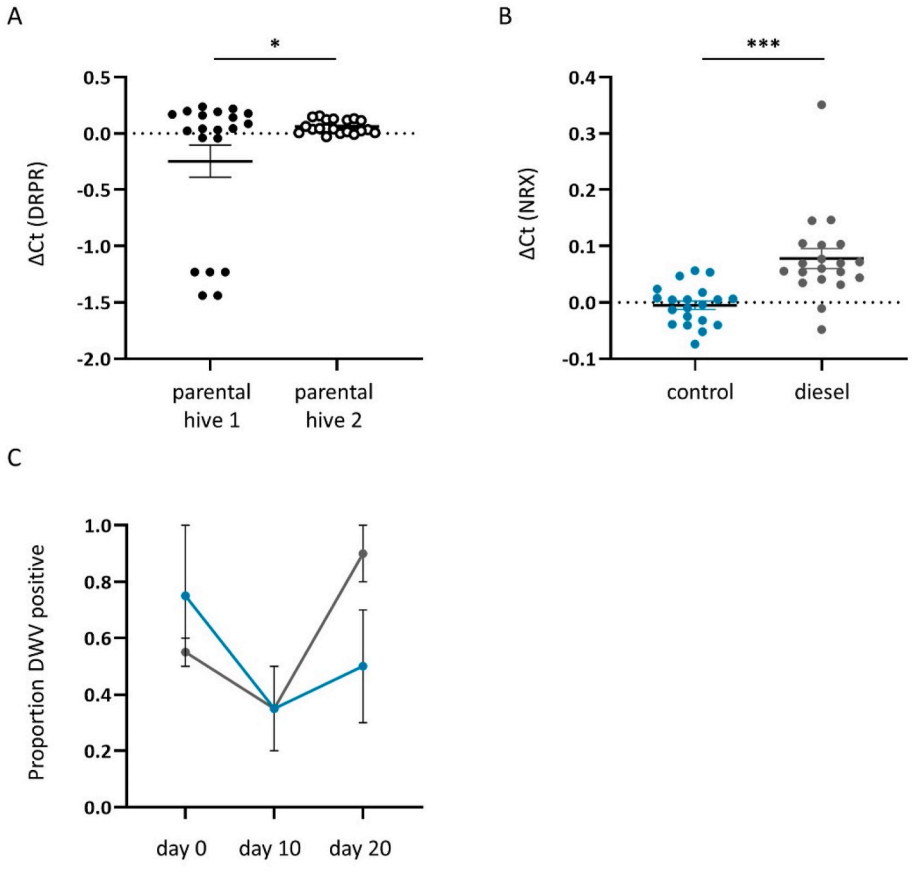

Fig. 1. Expression levels for drpr (A) and NRX1 (B) in the central nervous system of returning foragers, after 10 days of the repeated exposure experiment, shown as individual data points with mean values (±SE). The data shows significant effect of parental hive on DRPR expression (F1,36 = 4.35, P = 0.044) and a significant effect of treatment on NRX1 expression (F1,36 = 19.48, P = <0.001). Asterisks indicate significance levels *: p < 0.05 and ***: p < 0.001. Expression levels are shown relative to 0d group of corresponding hive/treatment and normalized against RPL8. (C) Interaction between time and treatment is shown for DWV prevalence as mean proportion of foragers sampled from two control (blue) and two diesel exhaust treated (grey) hives at three different timepoints (0d, 10d and 20d). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

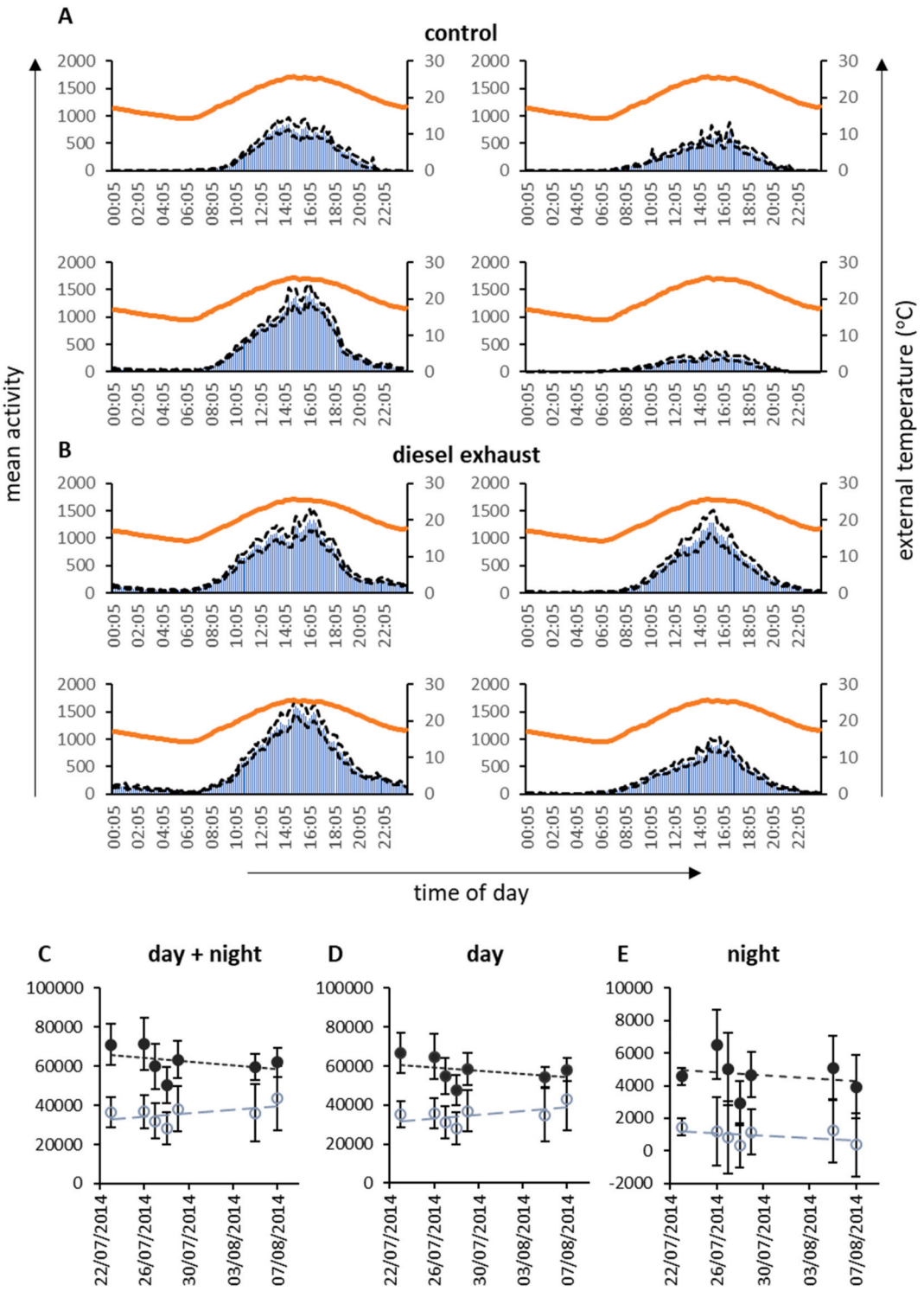

Fig. 2. Top (A–B): Mean honey bee counter unit total hive activity data for control (A) and diesel exhaust treated (B) hives over time during the experimental period. Blue bars = mean total number of bee movements (in and out) per 10-min period over the course of the experiment, black dotted lines are +ve and -ve standard errors; orange lines = ambient temperature (obtained from http://www.southamptonweather.co.uk/station located at 50° 53′ 58.96″ N 1° 23′ 43.69″ W). Bottom (C–E): Mean honey bee counter unit total hive activity data for control (blue, empty circle) and diesel exhaust treated (grey, full circle) hives (±SE) on specific days during the experimental period. Data shown is for: C) 24-h period; D) only daytime hours and E) only night-time hours. Dotted lines indicate linear regression lines of best fit for each treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

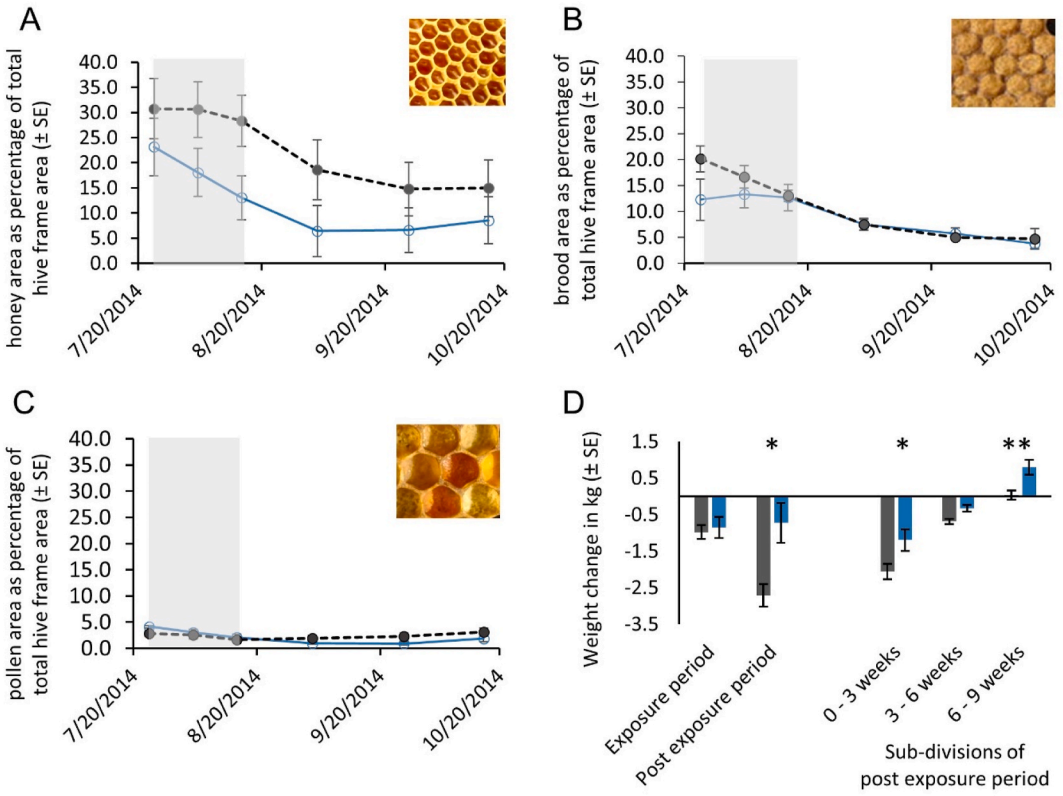

Fig. 3. Frame area filled with honey (A), brood (B) and pollen (C) as mean total frame hive area of control and diesel exhaust treated hives (±SE). Solid blue line = control hives, dotted grey line = treatment hives. Changes in hive weight in kg (±SE) of diesel exhaust treated hives (dark grey) and control hives (blue) between different time points, including the course of the exposure period, the nine-week period following directly after the exposure period and three-week subdivisions of this nine-week post-exposure period (D). Asterisks indicate significant differences between weights of control and diesel exhaust treated hives at: *P < 0.05, ** <0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

under the two treatments diverged in the actual species they collected over the course of the experiment and then returned to using similar flower species after the end of the experimental treatment period.

3.5. Hive product composition

Although frames were carefully distributed between nucleus hives at the beginning of the experiment, to ensure similar starting conditions between control hives and diesel exhaust treated hives, diesel exhaust treated hives started with similar but slightly higher weight and honey stores as well as a slightly higher amount of brood.

There was a significant effect of date on honey stored ($F_{4,24} = 31.47$, $P < 0.001$) but no effect of treatment ($F_{1,6} = 2.99$, $P = 0.14$) and no interaction between date and treatment ($F_{4,24} = 2.12$, $P = 0.11$, Fig. 3A). For brood there was a significant effect of date ($F_{4,24} = 23.96$, $P < 0.001$) but no effect of treatment ($F_{1,6} = 0.22$, $P = 0.66$) and no interactions between date and treatment ($F_{4,24} = 0.63$, $P = 0.65$, Fig. 3B). For pollen there was no effect of date ($F_{4,24} = 2.60$, $P = 0.06$) and no effect of treatment ($F_{1,6} = 1.15$, $P = 0.32$) and no interactions between date and treatment ($F_{4,24} = 1.65$, $P = 0.19$, Fig. 3C).

3.6. Hive weight

Changes in hive weight were calculated for the duration of the experiment and then for three-week intervals after the exposure period ended (early September and mid-October, see Fig. 3D). Over the course of the exposure period, both diesel exhaust treated and control hives lost weight but there was no significant difference in the rate of weight loss ($F_6 = 0.3$, $P = 0.74$). However, diesel exhaust treated hives exhibited greater weight loss during the nine-week post-exposure period ($F_6 = 4.6$, $P = 0.004$). When this nine-week post-exposure period was further subdivided into three-week measurement intervals, we observed a significant difference in weight loss between diesel exhuast treated and control hives in the first three weeks after the end of the exposure ($F_6 = 3.5$, $P = 0.01$) but not during the period between three and six weeks after the end of the exposure ($F_6 = 2.2$, $P = 0.07$). However, between six- and nine-weeks post-exposure, hives from both treatment groups started to re-gain weight and control hives put on significantly more weight than diesel exhaust treated hives ($F_6 = 3.9$, $P = 0.008$).

4. Discussion

This study provided a first investigation into variables pertaining to the fitness of honey bee hives and individual forager bees during the course of a three-week exposure period to diesel exhaust. During this period, many of the parameters investigated did not display significant changes above normal natural variation between hives repeatedly exposed to diesel exhaust and control hives. However, several effects of repeated short-term exposure to diesel exhaust were identified and furthermore, over an extended duration up to nine weeks after the exposure period, those hives that were exposed to diesel exhaust exhibited declines in key measures of hive success. These are the results of a single study in one location in a single year, with relatively moderate hive replication and therefore we would urge caution to be taken in interpreting the wider implications of these data. Nonetheless, they do provide novel insight into the effects that repeated exposure to low level air pollution has on honey bee colonies and individual foragers.

After 10 days of daily exposure to moderate levels of diesel exhaust, equivalent to or lower than would be commonly experienced across a day in a nearby urban centre (see Table S1), we observed an

upregulation of NRX1 expression in the CNS of foraging honey bees. Associative learning and memory formation are crucial mechanisms by which forager honey bees learn, locate and recall profitable foraging sites in the field (Menzel, 1993), and NRXs, which are trans-membrane cell adhesion molecules, facilitate the development and maintenance of synapses crucial for memory formation (Chen et al., 2011; Dean and Dresbach, 2006; Südhof, 2008). Functional analysis of NRX expression in honey bees has revealed that after successful Pavlovian conditioning trials, known as proboscis extension reflex (PER) trials in honey bees, NRX1 expression was upregulated in trained compared to untrained honey bees (Biswas et al., 2010). Furthermore, our previous findings demonstrated that honey bees acutely exposed to diesel exhaust exhibited impaired learning and memory of floral odours during such PER trials (Reitmayer et al., 2019). During the current study we observed that foragers from hives exposed to diesel exhaust showed upregulated expression of NRX1, which is indicative that these foragers were engaged in active learning and memory processes prior to our analyses. Considering hives from both treatments were co-located with access to the same resources, our results could point to an increased need for repeated learning of the same information in order to perform the same task as efficiently as the foragers from the control hives; however, the precise mechanisms that would result in such changes are unclear.

We further investigated whether repeated short-term exposure to diesel exhaust influenced the expression of drpr, a marker for insect glial cell activation. The draper signalling pathway is involved in the clearance of degraded axons following neuronal injury in the insect brain (Doherty et al., 2009; MacDonald et al., 2006) and is therefore a marker of CNS health. Our results did not suggest an effect of repeated short-term exposure to diesel exhaust on drpr expression, suggesting that such exposure did not result in increased neuronal injury across the time-period studied.

Molecular analysis of three common honey bee viruses (DWV, IAPV, BQCV) revealed no difference in prevalence of these viruses in foraging bees between diesel exhaust treated and control colonies. DWV infestation was previously found to correlate with winter losses in honey bee colonies (Highfield et al., 2009) and could therefore be an important factor in assessing a colony's fitness and likelihood to survive the winter. There was no difference in viral genome copy numbers in DWV positive foragers derived from diesel exposed hives and control hives. However, our data indicated that there is a significant interaction between exposure time and treatment, with a larger divergence of DWV prevalence between the treatment groups towards the end of the exposure period. Later time points would be needed to conclusively assess whether repeated short-term exposure to diesel exhaust would over time affect DWV prevalence.

Focusing upon the performance of whole hives, there were few effects observable during the three-week experimental exposure period, with mean total hive activity not differing between treatments and no significant effect of treatment on hive activity over time. However, although not statistically significant, there was an increase in daytime hive activity over the course of the experimental exposure period in control hives, and a decrease of hive activity in diesel exhaust treated hives. There were also very few differences between the composition of pollen carried by returning foragers from the different treatments.

During the exposure period both control and diesel exhaust treated hives lost weight, but again there were no differences between treatments in the rate at which weight was lost. In contrast, during the nine weeks after the experimental treatment we observed significant differences in hive weight between treatments, with diesel exhaust treated hives losing significantly more weight than control hives in the first three weeks after the treatment. Furthermore, between six and nine weeks after the treatment period control hives began to increase in weight at a significantly higher rate than the diesel exhaust exposed hives. Weight gain during this time of the year is mainly driven by the accumulation of nectar, which is necessary for winter survival; honey bee colonies reduce nectar to honey, providing their food source for the winter (Winston, 1987). In practice, managed honey bee colonies are also typically provided with supplementary food resources to use during the overwintering period, but the hives in the current experiment were not provided with such additional food supplies. Weight measurements were taken in the evening hours, just before the start of the daily exposure to diesel exhaust. We did not have a direct measure of worker numbers but at this time of the day, most workers are present in the hive and therefore the difference in weight is likely to be caused by the difference in honey stores and potentially also a difference in total worker numbers. However, towards the end of the season at our six-to nine--weeks post-exposure data point, due to honey bee colony seasonal behaviour, it is unlikely that any weight gain was caused by an increase in worker numbers. In addition, the area of brood stores during the previous three-week period (three-to six-weeks post exposure) remained constant for control hives, further indicating that weight increase is unlikely to have been due to an increase in worker numbers. In contrast, area of honey stores in the control hives increased towards the end of the recording period, but in the diesel exhaust treated hives the area of honey stores remained constant. Therefore, it is unlikely that the difference in hive weight was caused by a difference in brood or pollen stores, and so weight gain during this time is most likely to be attributable to accumulation of honey into the frames.

At three-to nine-weeks post-exposure, the worker bees present in the hive and thus the foragers responsible for collecting nectar would have included bees that were larvae during the experimental exposure period earlier in the season and therefore bees that were exposed to diesel exhaust throughout their larval development. It is possible that repeated short-term exposure to diesel exhaust during larval development may have resulted in longer-lasting effects on hive fitness by impacting those bees' ability as adults to gather enough nectar. However, the precise cause of the variations observed in changes in hive weight between the treatments is unknown because most of the parameters investigated here were only recorded during the three-week exposure period. As previously stated, this was a single study with relatively moderate replication, but the results obtained indicate that further investigation into the effects of repeated exposure to air pollution on honey bee colonies and foragers is warranted, and we would particularly encourage future studies to investigate these impacts over multiple years and elongated timescales.

5. Conclusion

Short daily exposures of honey bee hives to moderate concentrations of diesel exhaust resulted in the upregulation of a marker of synaptic plasticity in the CNS of forager bees, which suggests that these foragers from diesel exhaust treated hives may have had to invest more effort in learning, which is a critical component to foraging success. Whether this upregulation is indicative of an impairment of these individual's ability to fulfil their daily tasks as foragers was not clear from the results of this study and requires further investigation. However, the diesel exhaust treated colonies from which these forager bees were sampled saw significant reductions in hive weight and failed to regain weight later in the season. In contrast, control hives lost less weight and began to regain significantly more weight than the diesel exhaust treated hives. It was possible to attribute this weight gain in control hives to increases in honey stores, providing evidence that forager bees from the diesel exhaust treated hives were less efficient or successful in their foraging. These findings, in the absence of any obvious signs of colony disease or acute failure, suggest that a repeated short-term exposure to diesel exhaust could result in inefficient foraging behaviour, and that such a reduction in foraging behaviour could be linked to diesel exhaust exposure interfering with CNS functions of forager honey bees. This study provides support for previous findings on the effects of air pollution on a related species, the giant Asian honey bee, *Apis dorsata*, which demonstrated that increased air pollution resulted in decreases in bee survival and changes in metrics of physiology and gene expression

(Thimmegowda et al., 2020).

The pollutant concentrations that honey bee colonies were exposed to in this experiment were comparable to concentrations observed in urban environments (King's College London, 2020). Colonies placed next to busy roadways, such as along motorways, will be exposed to higher pollutant levels (e.g. mean hourly NO_x concentrations by the M25 motorway in Staines, UK, have been measured at 84.5 ppb (Sayegh et al., 2016)), and therefore the effects on CNS functions and foraging strategy might be stronger, particularly in the presence of additional stressors such as viral infections or exposure to pesticides. Ultimately, the effects observed in this study suggests that repeated short-term exposure to air pollution can act as an additional stressor on honey bee hives potentially inhibiting colony resilience and ultimately survival.

Credit author statement

Christine Reitmayer: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. Robbie Girling: Conceptualization, Methodology, Formal analysis, Writing – review & editing, Funding acquisition. Christopher Jackson: Methodology, Investigation. Tracey Newman: Conceptualization, Methodology, Investigation, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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