Acute exposure to urban air pollution impairs olfactory learning and memory in honeybees

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Statement of authorship

RJL, TP, PI, CM and DFH conceived the project; RJL collected the data; TP and PI conducted chemical analyses; RJL, TP, CM and DF wrote the manuscript. All authors gave final approval for publication.

Data accessibility statement

Should the manuscript be accepted, data supporting the results will be archived Dryad and the data DOI will be included within the article.

ABSTRACT

While the ecological effects of pesticides have been well studied in honeybees, it is unclear to what extent other anthropogenic contaminants such as air pollution may also negatively affect bee cognition and behaviour. To answer this question, we assessed the impacts of acute exposure to four ecologically relevant concentrations of a common urban air pollutant - diesel generated air pollution on honeybee odour learning and memory using a conditioned proboscis extension response assay. The proportion of bees that successfully learnt odours following direct air pollution exposure was significantly lower in bees exposed to low, medium and high air pollutant concentrations, than in bees exposed to current ambient levels. Furthermore, short- and long-term odour memory was significantly impaired in bees exposed to low medium and high air pollutant concentrations than in bees exposed to current ambient levels. These results demonstrate a clear and direct cognitive cost of air pollution. Given learning and memory play significant roles in foraging, we suggest air pollution will have increasing negative impacts on the ecosystem services bees provide and may add to the current threats such as pesticides, mites and disease affecting colony fitness.

Key words: Air pollution; honey bee; pollinator; proboscis extension reflex response; behaviour

INTRODUCTION

Bees are without doubt one of the most significant and economically important groups of pollinating insects worldwide (Klein et al. 2007, Potts et al. 2010). Alongside other pollinators, bees provide an ecosystem service essential to both agricultural productivity and the maintenance of wild plant communities (Potts et al. 2010). In Europe for example, 84% of crop species production depends on pollination (Williams 1994), whilst approximately 80% of wild plants rely specifically on insect pollinators (Kwak et al. 1998). It has been estimated that the global economic value of pollination is $190 billion (USD) per year (Gallai et al. 2009). Factors affecting the ability of bees to forage, therefore, not only have significant repercussions for the bees themselves and their colonies, but also for the essential pollination services these insects provide.

Bee foraging and ultimately their pollination services can be compromised by several factors including anthropogenic contaminants such as pesticides and heavy metals (Klein et al. 2017, Leonard and Hochuli 2017a). The widespread use of pesticides, for example, and in particular neonicotinoids have been directly implicated in reducing general motion (Williamson et al. 2014), waggle dancing (Eiri and Nieh 2012), foraging activity (Schneider et al. 2012), and olfactory learning

and memory (Williamson and Wright 2013). Pesticide use in agroecosystems is also suspected to have contributed towards declining pollinator populations and in particular North American and European honeybees (*Apis mellifera*) (Stokstad 2007, Potts et al. 2010). Continuing research on the sub-lethal effects of anthropogenic contaminants is crucial. Urban land cover expansion [i.e. between 430,000 km^2 and 12,568,000 km^2 by 2030 (Seto et al. 2011)], coupled with rising interest in urban agricultural activities (Eigenbrod and Gruda 2015), will likely increase the types and concentrations of anthropogenic contaminants bees encounter during their lifespan.

Urban air pollution is a pervasive and increasingly important form of anthropogenic contamination, known for its detrimental impacts on human health (e.g. exacerbation of chronic respiratory and cardiovascular diseases, decreased lung function, and premature mortality) (Kim et al. 2015). A significant source of urban air pollution in both developing and developed countries is the road transport sector (Gulia et al. 2015). In Delhi and Mumbai, 76%-90% of the ambient CO pollution and 66%-74% of the ambient NO_x pollution is attributed to vehicle use (Kamyotra et al. 2010). In the UK, road transport represents the largest source of urban air pollution in 92% of declared air quality management areas (Faulkner and Russell 2010). Despite this ubiquity, the ecological effects of traffic-related air pollution and in particular, direct impact on bees are relatively unknown (Girling et al. 2013, Leonard et al. 2017). Traffic-related air pollutants including ozone, hydroxyl radicals and nitrate radicals can indirectly affect bees by decreasing the detection distance over which odours, emitted by plants operate (McFrederick et al. 2008). Bees use such odours to locate, recognise and remember flowers of interest (Wright and Schiestl 2009). NO_x, a significant component of diesel exhaust, reduces the concentration of several oil seed rape floral volatile components (α-farnesene and phenylacetaldehyde), and their absence ultimately decreases flower recognition by honeybees (Girling et al. 2013, see also Leonard et al. 2019). Furthermore, when used to anesthetize bees for behavioural studies, CO_2, a component also present in traffic-related air pollution, directly impacts olfactory learning and memory (Erber 1976, Kirkerud et al. 2013); for instance, by reducing proboscis extension rate compared to other anaesthetization procedures (e.g. cooling on ice) (Kirkerud et al. 2013).

The extent to which the traffic-related air pollution encountered by bees directly affects foraging behaviours such as learning and memory is unknown. We therefore tested the effects of acute exposure to traffic-related air pollution on cognitive processes in the honey bee *Apis mellifera*. Acute exposure studies represent a good starting point in environmental risk assessment. After exposing bees to one of four ecologically relevant concentrations of diesel generated air pollution, we trained

individuals to learn a commonly encountered floral volatile, lavender, using the associative learning paradigm - olfactory conditioning of the proboscis extension response (PER) (Bitterman et al. 1983). This approach allowed us to specifically test the hypotheses that direct, acute exposure to traffic-related air pollution impairs olfactory learning and olfactory recognition across multiple memory phases.

METHODS

Honey bees

We used foraging adult worker *A. mellifera* from two disease free colonies started and maintained at the University of Sydney, Camperdown Campus (151.188512, -33.884924). Colonies were proximal to each other and maintained under the same environmental conditions prior to, and during the experimental period. During the summer of 2017 (i.e. December 1st – January 31st), on days when bees were active, we collected individual workers leaving both experimental hives between 6:00 and 7:00 GMT using an aspirator (approximately 30 individuals collected per day). These bees were kept in perforated containers (170mm x 120mm x 35mm) where they were exposed to air pollution as described below.

Air Pollution Treatments

We had four treatments representing incremental increases in air pollution, consistent with pulse event pollutant concentrations reported in street level urban and roadside environments in metropolitan cities for CO (Amorim et al. 2013), CO_2 (Kumar and Nagendra 2015), NO_2 (Irga et al. 2015), and total amount of emitted gas (i.e. tVOCs) (Varshney and Padhy 1998). The control treatment was ambient air (hence ambient pollution) exposure. To generate air pollution treatments, we created diesel fuel candles (volume: 18.15 mL; 18 mm height x 38 mm diameter) using a mixture of retail grade diesel fuel (Shell, Australia) and paraffin wax. Two formulations were created; the first representing a low pollutant dose concentration of diesel fuel to paraffin wax (25:75 ratio by volume), and the second representing a high pollutant dose (60:40 by volume). Candles were burned for different lengths of time to produce three pollution treatments: treatment 1 (low pollutant exposure): low pollutant dose candle burned for 30 seconds; treatment 2 (medium pollutant exposure): high pollutant dose candle burned for 15 seconds and treatment 3 (high pollutant exposure): high pollutant dose candle burned for 30 seconds.

To characterise the key components of the traffic-related air pollution generated by each treatment, we measured pollutant concentrations for a total of 5 minutes after burning each candle (Table 1) in

an exposure chamber containing pollutant measuring instruments (n=4). Total suspended particles (TSP) were recorded with a DustTrack II 8532 optical laser nephelometer (TSI, Shoreview, Minnesota); NO_2 concentrations were recorded using a GasAlert Extreme T2A-7X9 (BW Technologies, Canada); total volatile organic compounds (tVOC) were recorded using a portable photoionisation detector (ppbRAE; Rae Systems Inc., USA); CO and CO_2 concentrations were measured with a TSI IAQ-CALC portable infrared gas analyser (Table 1).

Exposure to air pollution treatments

We exposed bees to one of four air pollution treatments. In each case, bees (~30 individuals) were kept in perforated containers (170mm x 120mm x 35mm) where they could maintain flying behaviours and exposed to treatment for 5 min using a set-up similar to that described in Irga and Torpy (2017). In brief, during pollution exposure, bees were held in a sealed Perspex chamber (0.6 m x 0.6 m x 0.6 m; 216 L) fitted with ducting on one side (as an outlet) and a combustion chamber on the other. Air pollution treatments were generated by burning the respective diesel candle in the combustion chamber. Air movement from the combustion to the bee chamber was assisted by an impeller (FANTECH TEF-100 fan 16W) operating at 10% capacity, with impeller speed regulated by a single pole potentiometer. An additional fan was installed within the Perspex chamber to ensure bees were exposed to mixed air and a homogenous concentration of pollutants. To prevent pressure build-up, air was slowly removed from the chamber by a vacuum pump on the outlet ducting, operating at volumetric flow rate of 2.0×10^{-6} m^3s^{-1}.

Following exposure, bees were cooled in a freezer (-20°C) for ~5 min. Once bees ceased moving, we harnessed individuals in modified Eppendorf tubes so only their head was protruding, and their antennae and proboscis were free to move. This set-up was used for the subsequent behavioural assays (i.e. olfactory conditioning and recall tests). To ensure bees were at similar motivational states during behavioural assays, they were all starved for 3 hours, the period from initial capture to start of the assays. Before olfactory conditioning (15 min), we checked that the proboscis was intact by lightly touching the antennae with a toothpick soaked with 50% sucrose solution. Individuals who failed to extend their proboscis beyond their open mandibles were not used in the assays.

Absolute conditioning

Absolute conditioning aims to train organisms to recognise a stimulus (conditioned stimulus; CS) by associating that stimulus with a reward (unconditioned stimulus; US). The organism is said to recognise the stimulus when it displays the conditioned response (e.g. proboscis extension; PER). For

this experiment, we subjected harnessed bees to a standard absolute proboscis extension response (PER) conditioning protocol (Bitterman et al. 1983), by presenting one floral volatile (CS) coupled with 50% w/v sucrose solution (US). We chose to use the artificial floral volatile blend lavender (*Lavandula angustifolia L.*, Sigma Aldrich), as it representative of both a complex and commonly encountered floral volatile (Garbuzov and Ratnieks 2014). During conditioning we delivered the CS to the head of the bees through a 20 mL plastic syringe placed 2 cm in front of each bee. Into each syringe we placed a filter paper (0.5cm^2, grade GF/A; Whatman plc, Maidstone UK) impregnated with 1μl of the artificial lavender blend. For each bee, we used six conditioning trials per CS, and an inter-trial interval of 10 min between CS presentations (Menzel et al. 2001). Each conditioning trial lasted ~30 sec with CS presentation for 6 sec and reinforced with US 3 sec after onset of the CS. The US was delivered for 3 seconds using a toothpick soaked with 50% w/v sucrose solution, first briefly to the antennae and then for the remainder of the CS period, to the proboscis itself. During each of the six conditioning trials we recorded if bees extended their proboscis beyond their open mandibles before we delivered the US (i.e. during the first 3 seconds of CS delivery). To avoid contamination between CS presentations, we used an air extractor, placed 10 cm behind each test bee. Additionally, before volatile presentations, we acclimatized bees to test conditions by placing each bee in front of the air extractor for ~13 sec.

Recall tests

To evaluate short- and long-term memory formation in conditioned bees we tested the conditioned response (PER) 1 hr, 24 hrs and 48 hrs post conditioning respectively. During each recall test, we sequentially exposed bees to the CS. Recall tests resembled the conditioning trial (i.e. CS presentation for 5 seconds) but without the US. In each case we recorded the dichotomous response variable recognition which we classified as extension of the proboscis beyond the open mandibles. To ensure bees were not responding randomly during recall tests we excluded individuals that extended their proboscis in response to a control puff of air administered to the bee after recall tests using a clean plastic syringe. Following recall tests (approximately 1 pm daily), we fed bees to satiation (< ~10μl sucrose solution) using toothpicks soaked with 50% w/v solution. We classified individuals as satiated when they no longer reliably extended their proboscis after applying sucrose solution to the antennae. When we were not conducting recall tests, we kept bees on a cool dark shelf. We repeated the PER assay until we had over 30 test bees at the late long-term memory test point (see table Table 1 for replicate numbers).

Statistical analyses

Data from olfactory conditioning trials (i.e. learning) and memory recall tests were separately analysed using the generalized linear mixed model (GLMM) procedure with binomial distribution and logit link function. This analysis was chosen because data were binary and correlated [i.e. PER (yes/no) was recorded in the same individuals during conditioning trials 2 – 6 and recall tests 1 hour – 48 hours post conditioning]. Within each model, treatment (air pollutant concentration, N = 4), time (i.e. conditioning trial: N = 5 or recall test: N = 3) and their interaction term (i.e. treatment*time) were included as fixed factors. Source colony was also included as a random effect to account for potential differences in bees collected from each of two source colonies [note: we found no significant effect of colony on PER during conditioning ($F_{1, 1025}$ = 0.08, p = 0.77) or recall tests ($F_{1, 552}$ = 0.53, p = 0.46)]. The first conditioning trial was excluded from analysis because only bees naïve to lavender scent were used for conditioning assays. When appropriate, Bonferroni adjusted *post-hoc* tests were used to determine differences in response variables between levels of each fixed factor. To determine if treatment affected mortality at 48 hours, we used a generalized linear model (GLM) with binomial distribution and logit link function. All analyses were conducted in SPSS v 22 (IBM, Armonk, NY, USA).

RESULTS

Learning: Treatment ($F_{3, 1045}$ = 17.64, P < 0.001) and time (conditioning trial number, $F_{4, 1045}$ = 31.25, P < 0.001) but not their interaction ($F_{12, 1045}$ = 0.77, P = 0.68) significantly affected proboscis extension during olfactory conditioning. Post-hoc comparisons revealed significant differences between all pairwise treatment combinations except low and medium, and high and medium pollutant concentrations (Figure 1, Table 3). Proboscis extension following CS delivery, significantly increased with increasing conditioning trial number (Figure 1, Table 3).

Memory: Treatment ($F_{3, 542}$ = 25.15, P < 0.001) and time (conditioning trial number, $F_{2, 542}$ = 29.46, P < 0.001) but not their interaction ($F_{6, 542}$, 1045 = 0.77, P = 0.68) significantly affected proboscis extension during olfactory recall tests. Bees in the control group extended their proboscis significantly more following CS delivery than bees exposed to low, medium or high air pollution concentrations (Figure 1, Supplementary Table 3). Proboscis extension following CS delivery decreased significantly from 1 hour to 48 hours post conditioning (Figure 1, Table 4).

Mortality: We found no significant effect of treatment on mortality (Wald = 0.53, df = 3, p = 0.91). At 48 hours post conditioning, average mortality (±S.E.) was 30(±3)%, 28(±8)%, 28(±3)% 27(±4)% for the control and treatments low to high air pollution concentration respectively.

DISCUSSION

There was a strong negative effect of traffic-related air pollution on honeybee cognition; all pollutant concentrations exceeding current ambient levels caused significant reductions in olfactory learning and recognition across multiple memory phases. These results complement previous work investigating the indirect effects of traffic-related air pollution (Girling et al. 2013, Leonard et al. 2019, Lusebrink et al. 2015), and are the first to provide support for the hypothesis that a major air borne pollutant encountered by pollinators, diesel exhaust, may be directly capable of disrupting the odour recognition process that these insects rely on to locate, recognize and discriminate between floral food resources.

The proportion of bees exposed to the control treatment had significantly higher proboscis extension responses (93%) at conditioning trial 6 compared to the other treatments (71%, 59% and 55% for treatments low, medium, and high air pollution respectively). These findings are consistent with those investigating the effects of CO_2 anesthetization on bees, a common method used before harnessing individuals for cognitive assays [e.g. methods of Tan et al. (2015)]. Bees anesthetized with CO_2 have a significantly reduced proboscis extension response rate during conditioning (i.e. < 50%) compared to bees anesthetized using other methods including ice (Kirkerud et al. 2013). Furthermore, CO_2 narcosis post-training (e.g. < 30 min post-training) can lead to retrograde amnesia with bees responding to the CS significantly less after exposure to CO_2 for a prolonged time (i.e. 9 min) (Erber 1976).

The mechanism(s) by which treatment affected cognition remains to be determined. We suspect specific gasses and/or combinations of gasses found in traffic-related air pollution such as CO_2, $PM_{10/2.5}$ and heavy metals may be important. CO_2 for instance, can shift cellular pH levels, causing either de- or hyper-polarization of nerve cells (Brown and Berman 1970, Walker and Brown 1970). Indeed, electrophysiological recordings of extracellular neural activity in honeybee (*Apis mellifera carnica*) brains show an inhibitive effect of CO_2 and N_2 on clock spikes/activity (Erber 1976).

Interestingly, we found no significant differences in proboscis extension responses between low, medium and high air pollution concentrations during conditioning or at each recall test, suggesting a threshold-like (not incremental) effect of traffic-related air pollution on learning and memory. The question of whether bees would exhibit an acclimation response after chronic exposure to the concentrations used in this study (i.e. they respond similarly under control and high air pollution

treatments) is a fascinating topic for future research. We suspect that it is not unreasonable to predict bees encounter significantly different concentrations of traffic-related air pollution during and between foraging bouts (Leonard and Hochuli 2017b); traffic-related air pollution is highly spatially and temporally variable, and in urban areas, stop-start traffic flow coupled with the canyon effect of buildings create emission profiles that vary significantly (Namdeo et al. 1999).

Conclusions

Olfactory learning and memory in honeybees were impaired by acute exposure to traffic-related air pollutants. All concentrations exceeding current ambient levels (i.e. CO2: 398 ppm, NO: 0.90 ppm) affected learning and memory. These cognitive processes are essential to foraging behaviours including locating and discriminating between floral resources. Given we found a significant acute effect of traffic related air pollution, we suspect effects may be more serious after chronic exposure, a more likely scenario given the life expectancy of worker bees can range from less than 4 weeks in summer to more than 6 months in winter (Omholt and Amdam 2004). Further experiments investigating the physiological effects of traffic-related air pollution, as well as correlations between physiology and behaviour, will play a key role in understanding the underlying mechanisms involved.

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Compliance with ethical standards

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Conflict of Interest: All authors (R.J.L, T.J.P, P.I, C.M and D.F.H) declare no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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380 **Table 1. Sample sizes for conditioning trials and recall tests across treatments**

Air pollution concentration	Conditioning trial					Recall test		
	2	3	4	5	6	1-hour post conditioning	24 hours post conditioning	48 hours post conditioning
Control	59	59	59	59	59	59	48	42
Low	40	39	39	39	39	39	34	31
Medium	59	59	59	59	59	58	50	44
High	57	57	57	56	56	56	50	43

381

382 **Table 2.** Average concentration (± S.E.) for key air pollutants quantified in diesel generated pollution
383 for each treatment (n = 4 replicates per treatment)

Pollutant exposure treatment	PM (mg/m3)	CO (ppm)	CO_2 (ppm)	NO_2 (ppm)	TVOC (ppb)
Control: ambient	0.03 (±0.00)	2.28 (± 0.05)	398.00 (± 4.81)	0.90 (±0.00)	141.50 (± 2.53)
Treatment 1: low	2.49 (± 1.47)	2.98 (± 0.03)	462.75 (± 9.50)	1.00 (±0.00)	258.50 (± 40.69)
Treatment 2: medium	4.52 (± 1.16)	3.35 (± 0.12)	516.50 (± 9.08)	1.00 (±0.00)	604.50 (± 44.45)
Treatment 3: high	10.34 (± 1.42)	4.15 (± 0.16)	668.00 (± 32.07)	1.05 (±0.03)	823.50 (± 49.13)

384

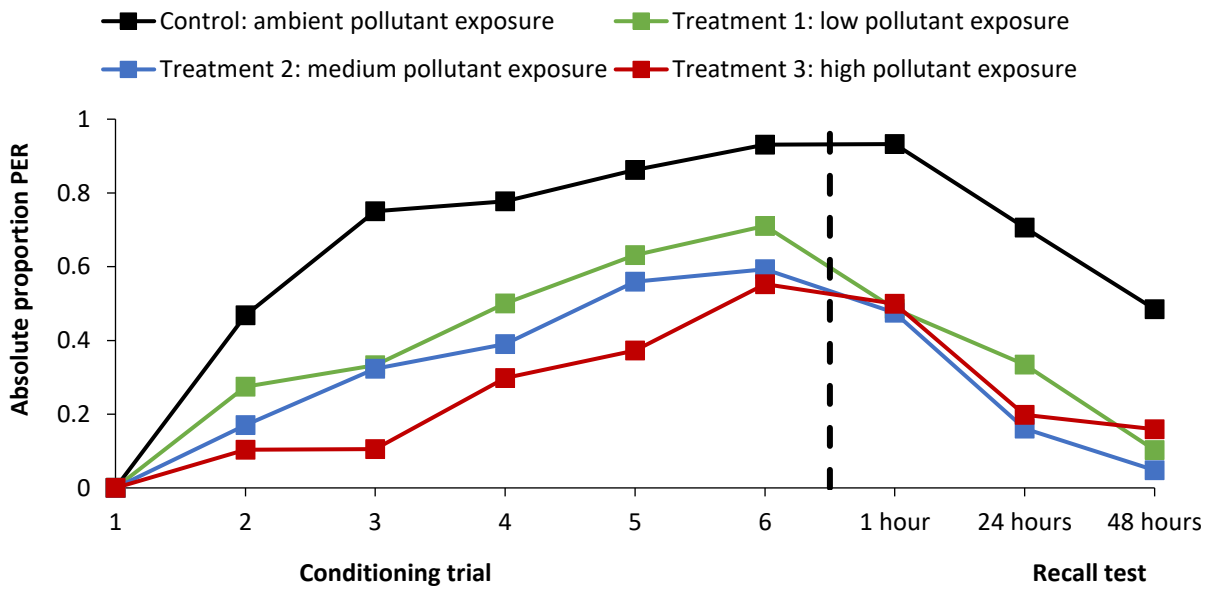

Figure 1. Proportion of bees (± S.E.) extending their proboscis in response to lavender, the conditioned stimulus during 6 conditioning trials and 1 hr, 24 hr and 48 hr post conditioning. Prior to conditioning bees were exposed to one of four acute air pollution treatments.

389 **Table 3.** Post-hoc comparisons for conditioning trials 2 through 6. P-values have been Bonferroni
390 adjusted

Pairwise contrast		t-value	Df	p-value
Air pollution concentration				
Control	Low	-3.59	1045	< 0.001
	Medium	-6.09	1045	< 0.001
	High	-10.26	1045	< 0.001
Low	Medium	-1.19	1045	0.23
	High	-3.00	1045	0.003
Medium	High	-2.052	1045	0.04
Time (conditioning trial)				
Conditioning trial 2	Conditioning trial 3	1.37	1045	0.17
	Conditioning trial 4	5.26	1045	< 0.001
	Conditioning trial 5	8.87	1045	< 0.001
	Conditioning trial 6	12.96	1045	< 0.001
Conditioning trial 3	Conditioning trial 4	3.53	1045	< 0.001
	Conditioning trial 5	6.81	1045	< 0.001
	Conditioning trial 6	10.01	1045	< 0.001
Conditioning trial 4	Conditioning trial 5	3.87	1045	< 0.001
	Conditioning trial 6	7.47	1045	< 0.001
Conditioning trial 5	Conditioning trial 6	3.59	1045	< 0.001

391

392 **Table 4.** Post hoc comparisons for recall tests 1 hour, 24 hours and 48 hours post conditioning. P-
393 values gave been Bonferroni corrected to account for multiple comparisons.

Pairwise contrast		t-value	Df	p-value
Air pollution concentration				
Control	Low	-7.36	542	< 0.001
	Medium	-10.14	542	< 0.001
	High	-8.42	542	< 0.001
Low	Medium	-1.55	542	0.12
	High	-0.001	542	0.99
Medium	High	-1.79	542	0.08
Time (recall test)				
1 hr post-conditioning	24 hrs post-conditioning	-5.61	542	< 0.001
	48 hrs post-conditioning	-9.32	542	< 0.001
24 hrs post-conditioning	48 hrs post-conditioning	3.41	542	0.001

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