Clearance of ingested neonicotinoid pesticide (imidacloprid) in honey bees (*Apis mellifera*) and bumble bees (*Bombus terrestris*)

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Abstract

BACKGROUND: Bees in agricultural landscapes are exposed to dietary pesticides such as imidacloprid when they feed from treated mass-flowering crops. Concern about the consequent impact on bees makes it important to understand their resilience. In the laboratory, we therefore fed adult worker bees on dosed syrup (125 μg L^{-1} imidacloprid, 98 μg kg^{-1}) either continuously or as a pulsed exposure and measured their behaviour (feeding and locomotory activity) and whole-body residues.

RESULTS: On dosed syrup, honey bees maintained much lower bodily levels of imidacloprid than bumble bees (< 0.2 ng *vs*. 2.4 ng imidacloprid per bee). Dietary imidacloprid did not affect the behaviour of honey bees but it reduced feeding and locomotory activity in bumble bees. After the pulsed exposure, bumble bees cleared bodily imidacloprid after 48 hours and recovered behaviourally.

CONCLUSION: We attribute the differential behavioural resilience of the two species to the observed differential in bodily residues. The ability of bumble bees to recover may be environmentally relevant in wild populations that face transitory exposures from the pulsed blooming of mass-flowering crops.

Keywords: detoxification, ecotoxicology, insecticide, oilseed rape, pulse exposure, recovery

1 INTRODUCTION

Neonicotinoid pesticides (e.g. imidacloprid, clothianidin and thiamethoxam) are widely used for the systemic protection of crops against biting and sucking insect pests [1]. Neonicotinoid residues pervade the roots and green tissues of treated plants[2] but they also appear at trace levels in the nectar and pollen of flowers, which bees consume [3]. In various laboratory and semi-field trials, dietary neonicotinoids can have harmful sublethal effects[4] on both honey bees (*Apis mellifera* L.) [5, 6] and bumble bees (*Bombus spp*.) [7, 8] , which has raised concern over the use of neonicotinoids across extensive areas of crops [9] and the potential threat to valuable pollination services for crops and wild plants [10]. Currently, the existence of other detrimental drivers, such habitat degradation and impacts from pathogens [10], and the lack of decisive field trials [5,11] leaves uncertainty over the relative importance of low-dose dietary exposures from neonicotinoid-treated mass-flowering crops such as oilseed rape (*Brassica napus* L.).

A further basis for concern is the length of time that bees are exposed to the pesticide in their diet. For example, each field of oilseed rape blooms for several weeks [12] and so some adult bees that forage on a treated crop's flowers could be exposed to dietary pesticide for their entire flightspan, which is about two or three weeks in bumble bees [13, 14] and one week in honey bees [15, 16]. If bees fail to clear ingested pesticide from their bodies, the persistence of even minute daily intakes could eventually build up to harmful levels over successive days. Furthermore, persistence compromises recovery in adult workers whose flight span intersects partially with the bloom of a mass-flowering crop so as extend beyond the crop's flowering. Fields of a mass-flowering crop like oilseed rape typically bloom in

synchrony across a landscape [12] and then flowering subsides, which causes neonicotinoid-exposed bees to shift their foraging to untreated wild flowers [17] and to thereby experience a 'pulsed' exposure. The onset of a pesticide-free diet could enable bees to recover by clearing pesticide from their systems unless the pesticide is persistent. Recovery is fairly rapid in other organisms following a pulsed exposure to imidacloprid. For example, feeding rates in coccinellid beetles (*Serangium japonicum*) [18] and aphids (*Myzus persicae*) [19] recovered within 24 h, egg production in whitefly (*Bemisia tabaci*) recovered after 48 hours [20] and the behavioural activities of aquatic larvae of *Chironomas* recovered within six days [21]. What is known about the persistence of ingested neonicotinoids in bees?

After a single meal of imidacloprid, honey bees clear the pesticide and its metabolites from their body within 24 hours [22] and clearance is achieved principally by metabolic degradation rather than by excretion of the parent compound in bees [23] and other insects [24]. The capacity for clearance may explain the recovery of daily food consumption by honey bee colonies after a four-day pulsed exposure to dietary imidacloprid [25]. However, the evolution of whole-body toxicant burdens has not previously been studied in bees. We therefore investigated levels of whole-body residues in bees that fed on a neonicotinoid-dosed diet continuously over a period of eight days. Additionally, we studied the rate of clearance and behavioural recovery after a pulsed exposure of several days, which enabled us to address a scientific controversy. Neonicotinoids are neurotoxic and the reversibility of their interactions with their target sites in the insect nervous system is contested [26, 27]. If an assimilated pesticide binds persistently to its target receptors, symptoms should persist after dietary exposure ceases. We therefore investigated the bodily levels of

ingested imidacloprid in honey bees during a pulsed exposure in conjunction with assays of behavioural recovery. Since equivalent information on bumble bees is lacking, we studied them in parallel.

2 METHODS

We collected newly eclosed worker honey bees from the brood of a single queen-right colony that was maintained at the University of Exeter. We obtained worker bumble bees (*Bombus terrestris* L.) from a domesticated colony (Koppert B.V., Berkel en Rodenrijs, Netherlands). Honey bees were placed in cages of 10 individuals (0.12 m x 0.10 m x 0.02 m) and bumble bees were placed individually in cages (0.07 m x 0.07 m x 0.035 m). Bees were maintained under semi-controlled conditions: temperature between 23 °C and 27 °C, relative humidity between 21% and 47%; and 12:12 hours of light:darkness. Bees fed *ad libitum* from syrup feeders. For further husbandry details see Cresswell *et al*.[28] For acclimatization, newly caged bees fed on undosed syrup for 24 h before the experimental exposures. We estimated the mean fresh mass of honey bees used in our study as 0.14 g (SE = 0.01, n = 6) and that of bumble bees as 0.19 g (SE = 0.03, n = 6).

Imidacloprid was obtained as a solution in acetonitrile (Dr. Ehrenstorfer, Augsburg, Germany). Acetonitrile was removed by evaporation using a vacuum concentrator (ScanSpeed MaxiVac Beta, Labogene Aps, Lynge, Denmark) and the imidacloprid was dissolved in the same volume of purified water before being mixed into feeder syrup (Attracker; Koppert B.V., Berkel en Rodenrijs, Netherlands) at a concentration

of 125 μg L^{-1}, or 98 μg kg^{-1}. This dosage was chosen for its known physiological efficacy [7,28] and not for environmental relevance.

We subjected bees to one of three treatments over eight days: the control group was fed undosed syrup; the continuous exposure group was fed dosed syrup throughout; the pulsed exposure group was fed dosed syrup for three days and undosed syrup thereafter. Each treatment group comprised three cages of 10 honey bees and 33 individually caged bumble bees. Each day, we measured syrup consumption and locomotory activity and collected three individuals for residue analysis (bumble bees were chosen at random and individual honey bees were collected haphazardly, one per cage).

To quantify locomotory activity, we observed each cage seven times at successive 30 minute intervals. On each occasion, each bee was scored as stationary or moving. For bumble bees, we calculated the proportion of the seven observations in which the bee was in motion. For honey bees, we calculated the proportion of bees in motion in each cage at each interval and then calculated the mean of these seven values. While scoring locomotion, the operator was unaware of the cage treatments.

To quantify the whole-body residue of imidacloprid in each collected bee, it was placed individually in a 2 ml Eppendorf tube (Sarstedt, Leicester, UK) and stored in a freezer at -80°C. To extract imidacloprid, a steel bead (0.4 mm diameter) and 25% methanol (1 ml) was placed in each vial and each was processed in a cooled tissue homogenizer for 5 mins at 25 rpm (TissueLyser, Qiagen, Crawley, UK). The homogenate was centrifuged (17000 g for 5 mins at 4°C) and we collected the

supernatant. For each species, the supernatants from the three bees collected on each day were pooled for LC-MS analysis.

The supernatant was diluted with an equal volume of 25% acetic acid and then subjected to solid phase extraction (SPE). The SPE column (Discovery DSC-18: bed weight = 50 mg; volume = 1 ml; Supelco, Bellefonte, Pennsylvania, USA) was conditioned with methanol (1 ml) and water (1 ml) before 650 µl of the sample was loaded. The column was washed with water (1 ml) followed by three elutions with methanol (200 µl). The combined methanol fractions were dried in the vacuum concentrator and stored at -80°C until LC-MS analysis.

For LC-MS analysis, each sample was re-suspended in a buffer of 25% methanol (400 µl) and passed through a 0.2 µm filter and spiked with a reference standard of 1 mg l^{-1} of deuterated imidacloprid (Dr. Ehrenstorfer GmbH, Augsburg, Germany). Each was then separated by liquid chromatography (Agilent 1200, Agilent Technologies, Santa Clara, CA, USA) using a reverse phase column (Agilent ZORBAX Rapid Resolution Eclipse Plus C18, Agilent technologies, Santa Clara, USA) interfaced *via* an electrospray ionisation source to a triple quadrupole mass spectrometer (Agilent 6410, Agilent Technologies, Santa Clara, CA, USA) and 10 µl of sample was injected. Mobile phase A was 0.1% formic acid + 5% acetonitrile and mobile phase B was 0.1% formic acid + 95% acetonitrile. The conditions of elution were: 0 min-0% B, 10 min-100% B, 12 min-100% B, 12.5 min-0% B. The flow rate was 0.3 ml min^{-1}. The source N_2 gas temperature was held at 350°C with a flow of 11 l min^{-1} and a nebulizer pressure of 35 psi. The capillary voltage was 4 kV. Fragmentor and collision energy voltages were 40 V and 20 V respectively.

Imidacloprid was identified and quantified by selected reaction monitoring (SRM) using the product ion *m/z* 209 derived from the precursor ion of *m/z* 256. The deuterated imidacloprid was detected using a precursor ion *m/z* of 260 and a product ion *m/z* of 213. The instrument response was linear between 10^{-2} ng and 1 ng imidacloprid. The amount of imidacloprid in the samples was estimated from the relative peak areas of unlabelled and deuterated imidacloprid in SRM chromatograms. We also adjusted for the recovery rate of the extraction method, which was quantified in a pilot trial in which known concentrations of deuterated and unlabelled imidacloprid were added to homogenates from undosed bees before performing the extraction protocol. The recovery rate from honey bee homogenate was 64% (SE = 1.1%, n = 3) and 52% (SE = 2.5%, n = 3) from bumble bee homogenate.

The biological half-life of assimilated imidacloprid (T_{half}) in bumble bees was estimated as $T_{half} = \ln(2)/k_e$ where k_e is the elimination constant. The elimination constant [22] is given by: $k_e = [\ln(C_1)-\ln(C_2)]/ (t_2-t_1)$ where C_1 and C_2 are the toxicant's concentration in the bee at times t_1 and t_2 respectively in the post-dose phase of the pulsed exposure. The level of detection (LOD) was given by: $LOD = C_0 + 3 \times SE(C_0)$, where C_0 is the mean level of imidacloprid detected in the negative control samples and $SE(C_0)$ is the standard error of this value [29]. The level of quantification (LOQ) was given by: $LOQ = C_0 + 10 \times SE(C_0)$. We calculated daily clearance rate (%) as $C = 100*[1 - R_D/(I_D + R_{D-1})]$, where R_D denotes the mean whole-body residue on a given day, I_D denotes the amount of toxicant ingested on that day and R_{D-1} denotes the whole-body residue level on the previous day. We calculated R_D as the amount of imidacloprid per dosed bee minus the amount per undosed bee. For the statistical

analysis of behavioural effects, we calculated the average response of each experimental replicate across the exposure period (e.g. each cage yielded a single value of the average daily syrup consumption per bee).

3 RESULTS

In the LC-MS analyses, the LOD was 0.15 ng of imidacloprid per individual for honey bees and 0.10 ng for bumble bees. The LOQ was 0.21 ng for honey bees and 0.16 ng for bumble bees.

Individual honey bees that fed on dosed syrup for eight days ingested a mean of 2.2 ng d^{-1} of imidacloprid (i.e. a total of 17.4 ng) and maintained bodily residues of approximately 0.2 ng (1.4 ng g^{-1}), which were not distinguishable from residues in bees that fed on undosed syrup (paired *t*-test, $t = 1.34$, df = 7, $P > 0.1$; Fig 1a). The daily clearance is therefore estimated as $C \approx 100\%$. Mean *per capita* daily rates of feeding (one-tailed *t*-test, $t = 0.39$, $df = 4$, $P = 0.36$) and mean level of activity (one-tailed *t*-test, $t = 0.29$, $df = 4$, $P = 0.39$) did not differ between dosed and undosed bees (Fig 2).

Based on the mass of syrup consumed and the concentration of imidacloprid, we estimate that individual bumble bees that fed on dosed syrup for eight days ingested a mean of 6.7 ng d^{-1} of imidacloprid (i.e. a total of 53.8 ng). From the fourth to the eighth days of feeding on dosed syrup, bumble bees maintained bodily residues of approximately 2.4 ng (12.9 ng g^{-1}), which was higher than the level in undosed bees (paired *t*-test, $t = 10.24$, df = 4, $P < 0.001$; Fig 1b). The daily clearance rate in

bumble bees is therefore estimated as C = 88% on the first day of ingesting imidacloprid and $C \approx 68\%$ thereafter. Bodily residues were higher in bumble bees than honey bees (paired t-test, $t = 9.77$, df = 7, $P < 0.001$). When imidacloprid was removed from their diet, bumble bees eliminated bodily residues after 48 h (Fig 1b) and the biological half life of imidacloprid was T_{half} = 10.3 hours. Dietary imidacloprid reduced mean daily rates of feeding (one-tailed t-test, $t = 3.94$, df = 53, $P < 0.001$) and mean daily locomotory activity (one-tailed t-test, $t = 3.05$, df = 57, $P = 0.002$) in bumble bees. Bumble bees in the pulsed exposure became more active than the undosed controls the day after the toxicant was removed from their diet (t-test, $t = 4.79$, df = 20, $P < 0.001$; Fig 3b) and their feeding rate appeared to recover (Fig 3a).

Discussion

As previously [28], we found that imidacloprid at a dietary concentration of approximately 100 parts per billion reduced the rates of feeding and locomotory activity in adult worker bumble bees but not honey bees. We attribute this difference to the observed differential in whole-body residues that was evident during the dietary exposure. Specifically, individual honey bees continuously metabolized or otherwise eliminated their daily intake of approximately 2 ng day^{-1}, which is almost half the LD_{50} (48 h oral $LD_{50} \approx 4.5$ ng) [5]. In contrast, bumble bees cleared less than 70% of assimilated imidacloprid each day and therefore exhibited a higher level of whole-body imidacloprid. In bumble bees, the correspondence between behavioural recovery and the clearance of bodily residues after dietary intake ceased supports our interpretation that whole-body residues reflect the relative levels of toxicant at the target site, but not necessarily the absolute levels. Specifically, we recognize that

the higher whole-body residue levels in bumble bees may have been caused in part by newly ingested syrup in the bee's relatively large honey stomach. Also, our observations do not exclude the possibility that greater target site-sensitivity contributed to the more severe impact on bumble bees but there is currently no evidence to support this, although such variation is known among other insect species [30,31].

We observed that individual bumble bees ingested approximately three times more imidacloprid than individual honey bees over the eight day exposure. Indeed, the greater feeding rate of bumble bees may be the principle cause of their susceptibility rather than a deficiency in detoxification capacity. Once the levels of bodily residues had stabilized, individual bumble bees were capable of clearing about three times more imidacloprid per day than honey bees (i.e. about 7 ng of imidacloprid per day compared to 2 ng per day). Consequently, the relatively high levels of bodily residues in bumble bees were apparently sustained by their relatively high rates of ingesting toxicant. We cannot fully explain why bumble bees consumed so much more syrup than honey bees. The higher food consumption of bumble bees is not attributable solely to body size because their mass was only 40% greater than that of the honey bees in our study. Also, we cannot attribute it to a putative energetic cost of detoxifying imidacloprid because even undosed bumble bees consumed six times more syrup than undosed honey bees. Instead, we speculate that bumble bees metabolized the syrup while maintaining relatively high body temperatures [32]. We therefore hypothesize that their high energy requirement may predispose bumble bees to impacts from toxicants in nectar.

We found no evidence that imidacloprid accumulated persistently in either species. In our experiment, adult honey bees cleared imidacloprid at the rate of ingestion, which consistent with the previously reported biological half life of about four hours [23]. Even in bumble bees, we found that bodily residues equilibrated and that the biological half life of imidacloprid was only approximately 10 hours. Our findings have implications for investigators of environmentally relevant impacts of neonicotinoid pesticides on bees. Specifically, it is unrealistic to apply the daily aggregate dose in a single meal [6] because it could have a stronger effect than if the same amount of toxicant were ingested gradually over the course of the day, as would happen if the bee foraged normally on flowers with residues in nectar and pollen.

Even though bumble bees were affected by dietary imidacloprid, they nevertheless cleared the toxicant from their bodies within 48 hours and recovered behaviourally when fed undosed syrup. This finding undermines previous assertions that imidacloprid irreversibly blocks nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insects [26, 33]. Similarly, a wide range of physiological evidence contraindicates irreversibility, as follows. The nicotinic acetylcholine receptors (nAChRs) in the insect nervous system are ligand-gated ion channels that are normally activated by a natural neurotransmitter, acetylcholine, but neonicotinoids also act as ligands [34] and so disrupt coordinated nerve activity. The electronegative pharmacophore of neonicotinoids (a nitro or cyano group) interacts with the binding pocket of the pentameric nAChRs through residues in various polypeptide loops that are upstream of loop B [30], within loop C of α subunits [35], and in loop D of β subunits [36, 37]. These interactions are not covalent and instead involve

hydrogen bonds [36], electrostatic cation-π interactions [38, 39] and Van Der Waals interactions [35], which are all relatively weak and therefore reversible. This potential for reversibility is realized in bath-perfusion experiments on isolated neurones [40] that show that the depolarisation caused by bathing the cell in imidacloprid is rapidly reversed once the imidacloprid is washed away. Additionally, competitive-displacement experiments show that radio-labelled imidacloprid is displaced from the binding pocket of nAChRs by acetylcholine itself [31]. The capacity for displacement is confirmed by bath-perfusion electrophysiology, where the depolarising effect of imidacloprid can be reversed by increasing the concentration of the natural neurotransmitter, acetylcholine [34].

The bees' capacity for recovery lends significance to the pulsed blooming of mass-flowering crops like oilseed rape. Our findings suggest that bees may recover from exposure to dietary imidacloprid once the flowering of the pesticide-treated crop subsides but further research is required to evaluate the role of post-exposure recovery under environmentally realistic conditions.

We quantified only imidacloprid but we infer that its metabolic derivatives [23] were cleared by bees over a similar timescale as their parent compound for the following reasons. In honey bees, there was no indication of any dose-dependent effect on behaviour. In bumble bees, behavioural alteration and recovery corresponded to bodily levels of imidacloprid, which contraindicates the proposition that toxic derivatives of imidacloprid have effects separable from those of the parent compound [23]. Because of these considerations, we do not attribute the post-dose increase in the activity level of bumble bees in the pulsed exposure treatment to the

delayed production of a stimulatory derivative. Instead, we propose two explanations. First, withdrawal of a cholinergic agonist can increase sensitivity of serotinergic neurones [41], which are a type that influences flight activity in insects [42]. Potentially, a similar mechanism of sensitisation may have increased post-exposure locomotory activity in our experimental bees. Alternatively, the heightened activity may be a bee's response to previous intoxication. Social insects such as ants and honey bees exhibit altruistic self-removal [43] whereby diseased individuals leave the colony. We therefore speculate that the heightened activity of bumble bees post-exposure was the result of an intrinsic adaptive response, namely attempted self-removal but this conjecture needs to be substantiated by a demonstration that bumble bees exhibit this behaviour.

Bumble bees are affected by dietary concentrations of imidacloprid that are far lower than we used in our present experiment [7, 28, 44], which suggests that incomplete clearance of continuously ingested toxicant occurs irrespective of the level of dietary exposure. To us, it is surprising that bumble bees are affected by rates of ingestion in the region of 1 ng of imidacloprid per day [7] even though they are capable of clearing 5 ng daily. One possible explanation is that the metabolic degradation of imidacloprid is not fast enough to prevent low levels of toxicant reaching target sites, but further research is required to evaluate this speculation.

Other insects recover from the effects of imidacloprid after pulsed exposure [18,19,45] but we are the first to demonstrate that behavioural recovery from intoxication coincides with bodily clearance of the toxicant. Our observation supports the hypothesis that the interaction between imidacloprid and its target receptors in the

bee nervous system is in large part reversible and not persistent as some have asserted [26, 33]. In separate experiments, we have also observed recovery of brood production by bumble bee (*B. terrestris*) queens after a pulsed exposure to dietary imidacloprid of 14 days at dosages up to 125 μg L^{-1} (Laycock and Cresswell, unpublished). We therefore anticipate that recovery, in whole or in part, will generalize to various endpoints in this species.

Our observation that bumble bees can rapidly clear imidacloprid once ingestion ceases lends significance to the pulsed blooming patterns of bee-attractive mass-flowering crops such as oilseed rape. Specifically, the crop's flowering is typically fairly synchronous across a landscape [12] and the impact of imidacloprid-treated crops on bumble bees may be ameliorated if they recover as the blooming subsides by switching to a diet of untreated wild flowers [17]. Further research is required to establish whether the behavioural recovery that we observed under laboratory conditions means that bumble bees recover their full performance under ecologically relevant conditions.

Acknowledgements.
The work reported here conforms to the regulatory requirements for animal experimentation in the UK and has been approved by the Biosciences Ethics Committee at the University of Exeter.

FIGURE LEGENDS

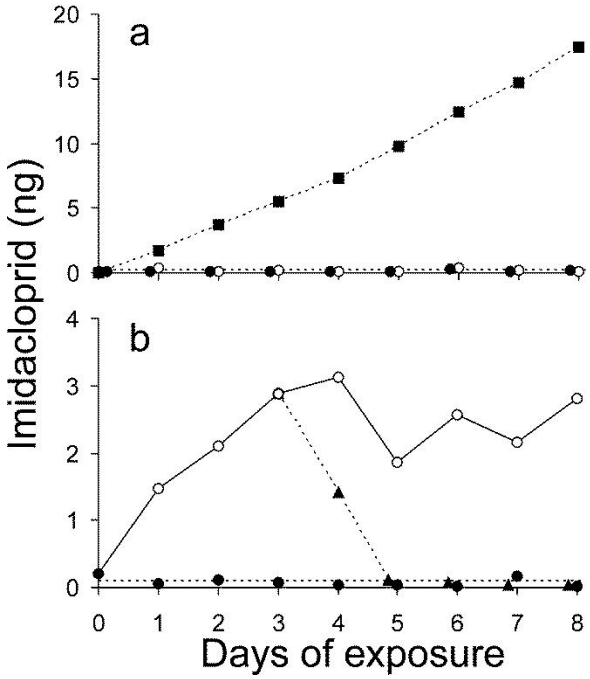

Figure 1. Imidacloprid budgets in bees over time in an eight day exposure experiment. Panel (a): interpolated square symbols denote the cumulative mass of imidacloprid consumed per honey bee (ng). Other symbols denote whole-body residues (ng) in undosed honey bees (filled circles) and dosed honey bees (open circles). The dashed horizontal line indicates the mean whole-body residue in undosed controls calculated across the eight day exposure. Panel (b): whole-body residues (ng) in undosed bumble bees (filled circles) and dosed bumble bees (open circles). Triangles denote the whole-body residues in the pulsed exposure treatment after dosing ceased on day 3. The dashed horizontal line indicates the mean whole-body residue in undosed controls calculated across the eight day exposure. Points are interpolated for inspection purposes only and some values are displaced slightly in the x-dimension for ease of inspection. Imidacloprid was assayed in a single pooled homogenate of three individual bees collected from each dose on each day.

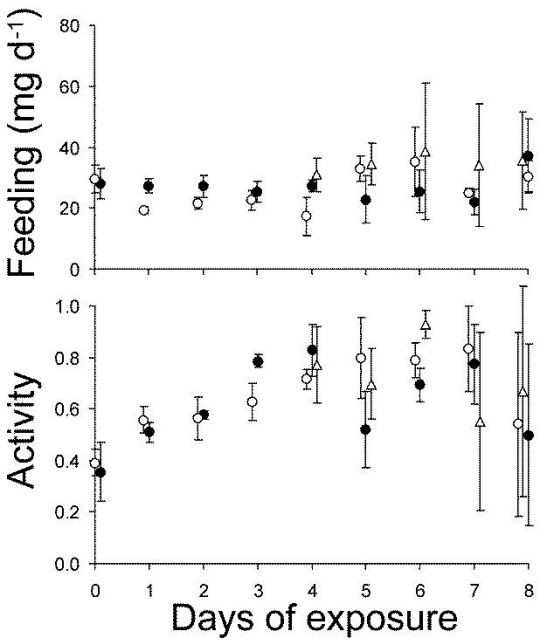

Figure 2. Behavioural responses (daily feeding rate and locomotory activity) of honey bees over time in an eight day exposure experiment. Panel (a): mass of syrup consumed per bee per day (mg). Panel (b): mean proportion of observations when the individual bee was in motion. Filled circles denote the undosed control treatment; open circles denote the dosed treatment; triangles denote the pulsed exposure treatment after dosing ceased on day 3. Responses on day = 0 are pre-experimental levels. Error bars = 1 SE. Some values are displaced slightly in the *x*-dimension to reveal their error bars.

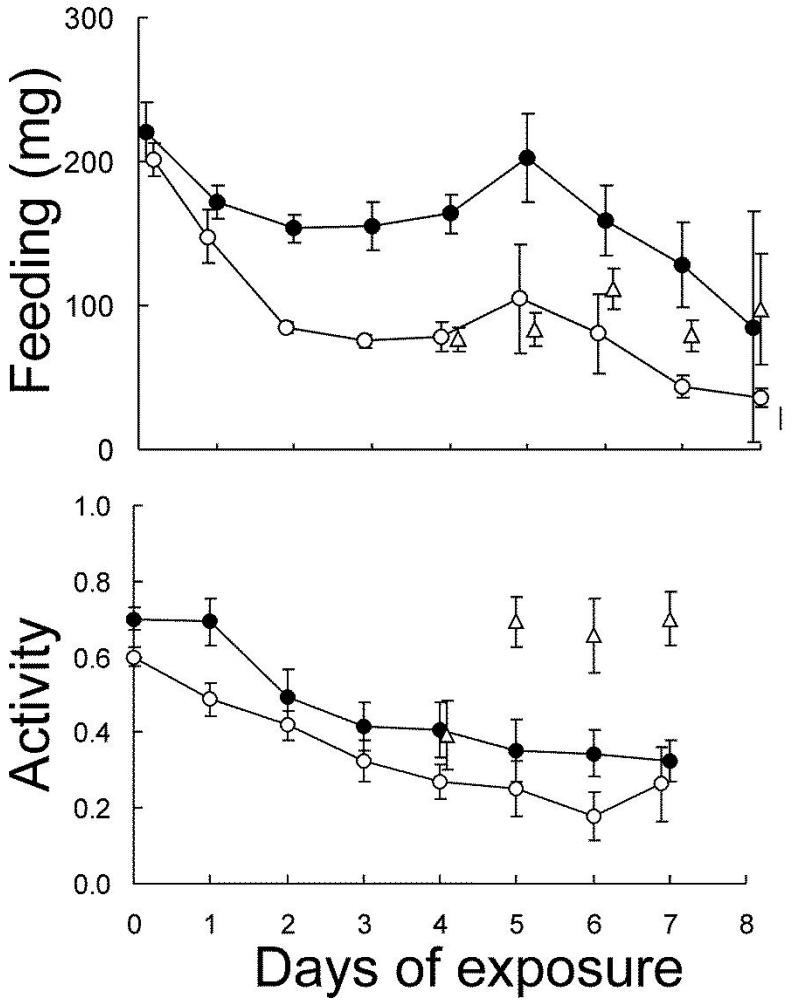

Figure 3. Behavioural responses (daily feeding rate and locomotory activity) of bumble bees over time in an eight day exposure experiment. Panel (a): mass of syrup consumed per bee per day (mg). Panel (b): mean proportion of observations when the individual bee was in motion. Filled circles denote the undosed control treatment; open circles denote the dosed treatment; triangles denote the pulsed exposure treatment when dosing ceased after day 3. Error bars = 1 SE. Points are interpolated for inspection purposes only and some values are displaced slightly in the x-dimension to reveal their error bars.

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