Research Article

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Clearance of ingested neonicotinoid pesticide (imidacloprid) in honey bees (*Apis mellifera*) and bumblebees (*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, the authors therefore fed adult worker bees on dosed syrup (125 μg L^{-1} of imidacloprid, or 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 bumblebees (<0.2 ng versus 2.4 ng of imidacloprid per bee). Dietary imidacloprid did not affect the behaviour of honey bees, but it reduced feeding and locomotory activity in bumblebees. After the pulsed exposure, bumblebees cleared bodily imidacloprid after 48 h and recovered behaviourally.

CONCLUSION: The differential behavioural resilience of the two species can be attributed to the observed differential in bodily residues. The ability of bumblebees 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 bumblebees (*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 as habitat degradation and impacts from pathogens,[10] and the lack of decisive field trials[5,11] have both left 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 for which 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 flight span, which is about 2 or 3 weeks in bumblebees[13,14] and 1 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 to extend beyond the crop's flowering. Fields of a mass-flowering crop such as 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 thereby to 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 h[20] and the behavioural activities of aquatic larvae of *Chironomas* recovered within 6 days.[21] What is known about the persistence of ingested neonicotinoids in bees?

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After a single meal of imidacloprid, honey bees clear the pesticide and its metabolites from their body within 24 h,[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 4 day pulsed exposure to dietary imidacloprid.[25] However, the evolution of whole-body toxicant burdens has not previously been studied in bees. Therefore, an investigation was made of the levels of whole-body residues in bees that fed on a neonicotinoid-dosed diet continuously over a period of 8 days. Additionally, the rate of clearance and behavioural recovery after a pulsed exposure of several days was studied, which made it possible 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. An investigation of the bodily levels of ingested imidacloprid in honey bees during a pulsed exposure was therefore carried out, in conjunction with assays of behavioural recovery. As equivalent information on bumblebees is lacking, they were studied in parallel.

2 METHODS

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

Imidacloprid was obtained as a solution in acetonitrile (Dr Ehrenstorfer GmbH, 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, The 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.

Bees were subjected to one of three treatments over 8 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 3 days and undosed syrup thereafter. Each treatment group comprised three cages of ten honey bees and 33 individually caged bumblebees. Each day, syrup consumption and locomotory activity were measured, and three individuals were collected for residue analysis (bumblebees were chosen at random, and individual honey bees were collected haphazardly, one per cage).

To quantify locomotory activity, each cage was observed 7 times at successive 30 min intervals. On each occasion, each bee was scored as stationary or moving. For bumblebees, the proportion of the seven observations in which the bee was in motion was calculated. For honey bees, the proportion of bees in motion in each cage at each interval was calculated, and then the mean of these seven values was taken. 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) were placed in each vial, and each was processed in a cooled tissue homogeniser for 5 min at 25 rpm (TissueLyser; Qiagen, Crawley, UK). The homogenate was centrifuged (17 000 g for 5 min at 4 °C), and the supernatant was collected. 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, PA) 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 resuspended in 25% methanol (400 μL), 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) using a reverse-phase column (Agilent ZORBAX Rapid Resolution Eclipse Plus C18; Agilent Technologies, Santa Clara, CA) interfaced via an electrospray ionisation source to a triple quadrupole mass spectrometer (Agilent 6410; Agilent Technologies, Santa Clara, CA), 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 with 0% B, 10 min with 100% B, 12 min with 100% B and 12.5 min with 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 nebuliser pressure of 35 psi. The capillary voltage was 4 kV. The fragmentor and collision energy voltages were 40 V and 20 V respectively. Imidacloprid was identified and quantified by selected reaction monitoring (SRM) using a product ion m/z of 209 derived from a precursor ion m/z of 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} 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. Also, adjustment was made 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 that from bumblebee homogenate was 52% (SE = 2.5%, $n = 3$).

The biological half-life of assimilated imidacloprid (T_{half}) in bumblebees 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 limit of detection (LOD)

was given by: LOD = $C_0 + 3 \times SE(C_0)$, where C_0 is the mean limit 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 = $C_0 + 10 \times SE(C_0)$. The daily clearance rate (%) was calculated 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. Residue R_D was calculated as the amount of imidacloprid per dosed bee minus the amount per undosed bee. For the statistical analysis of behavioural effects, the average response of each experimental replicate across the exposure period was calculated (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 bumblebees. The LOQ was 0.21 ng for honey bees and 0.16 ng for bumblebees.

Individual honey bees that fed on dosed syrup for 8 days ingested a mean of 2.2 ng day^{-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 was 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, it was estimated that individual bumblebees that fed on dosed syrup for 8 days ingested a mean of 6.7 ng day^{-1} of imidacloprid (i.e. a total of 53.8 ng). From the fourth to the eighth days of feeding on dosed syrup, bumblebees 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 bumblebees was therefore estimated as $C = 88\%$ on the first day of ingesting imidacloprid and $C \approx 68\%$ thereafter. Bodily residues were higher in bumblebees than in honey bees (paired *t*-test: $t = 9.77$, df = 7, $P < 0.001$). When imidacloprid was removed from their diet, bumblebees eliminated bodily residues after 48 h (Fig. 1b) and the biological half-life of imidacloprid was $T_{half} = 10.3$ h. 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 bumblebees. Bumblebees 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).

4 DISCUSSION

As previously,[28] it was found that imidacloprid at a dietary concentration of approximately 100 ppb reduced the rates of feeding and locomotory activity in adult worker bumblebees but not in honey bees. This difference is attributed to the observed differential in whole-body residues that was evident during the dietary exposure. Specifically, individual honey bees continuously metabolised 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, bumblebees cleared less than

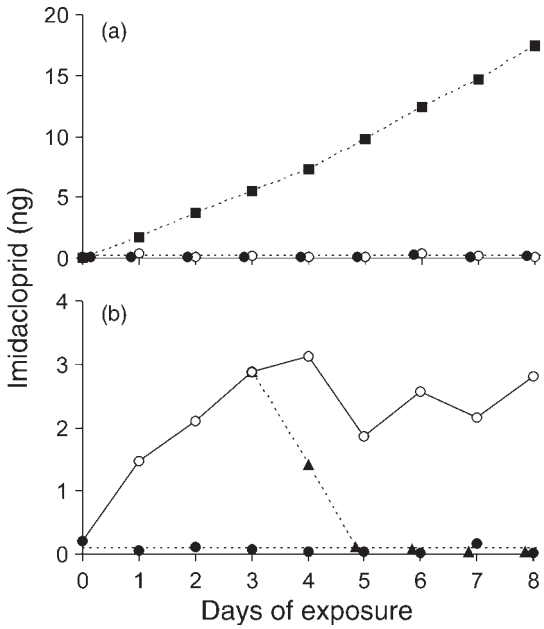

Figure 1. Imidacloprid budgets in bees over time in an 8 day exposure experiment. Panel (a): interpolated square symbols denote the cumulative mass of imidacloprid consumed per honey bee (ng). Other symbols denote whole-body residues (ng) in undosed honey bees (filled circles) and dosed honey bees (open circles). The dashed horizontal line indicates the mean whole-body residue in undosed controls calculated across the 8 day exposure. Panel (b): whole-body residues (ng) in undosed bumblebees (filled circles) and dosed bumblebees (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 8 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.

70% of assimilated imidacloprid each day and therefore exhibited a higher level of whole-body imidacloprid. In bumblebees, the correspondence between behavioural recovery and the clearance of bodily residues after dietary intake had ceased supports the authors' interpretation that whole-body residues reflect the relative levels of toxicant at the target site, but not necessarily the absolute levels. Specifically, it is recognised that the higher whole-body residue levels in bumblebees may have been caused in part by newly ingested syrup in the bee's relatively large honey stomach. Also, observations do not exclude the possibility that greater target-site sensitivity contributed to the more severe impact on bumblebees, but there is currently no evidence to support this, although such variation is known among other insect species.[30,31]

It was observed that individual bumblebees ingested approximately 3 times more imidacloprid than individual honey bees over the 8 day exposure. Indeed, the greater feeding rate of bumblebees may be the principle cause of their susceptibility rather than a deficiency in detoxification capacity. Once the levels of bodily residues had stabilised, individual bumblebees were capable of clearing about 3 times more imidacloprid per day than honey bees (i.e. about 7 ng day^{-1} as opposed to 2 ng day^{-1}). Consequently, the relatively high levels of bodily residues in bumblebees were apparently sustained by their relatively high rates of ingesting toxicant. The fact that bumblebees consumed so much more syrup than honey bees cannot be fully explained.

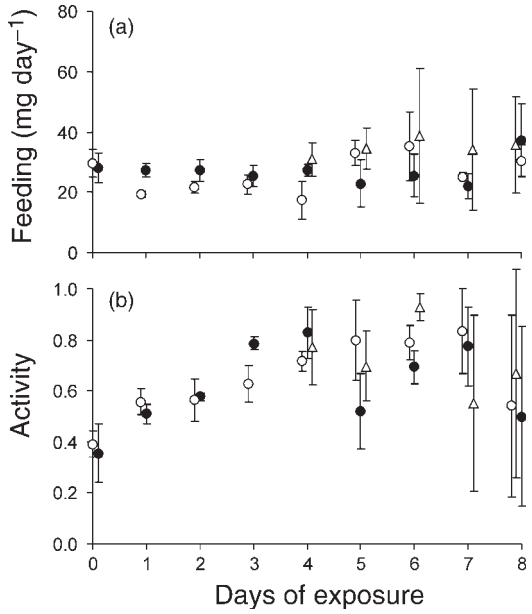

Figure 2. Behavioural responses (daily feeding rate and locomotory activity) of honey bees over time in an 8 day exposure experiment. Panel (a): mass of syrup consumed per bee per day (mg). Panel (b): mean proportion of observations when the individual bee was in motion. Filled circles denote the undosed 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.

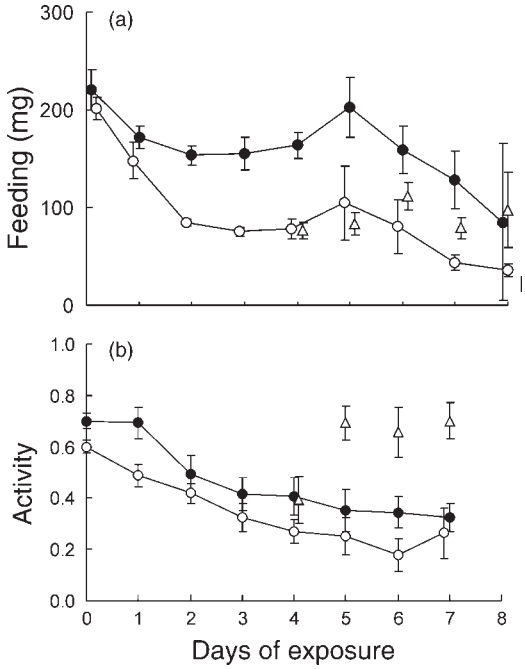

Figure 3. Behavioural responses (daily feeding rate and locomotory activity) of bumblebees over time in an 8 day exposure experiment. Panel (a): mass of syrup consumed per bee per day (mg). Panel (b): mean proportion of observations when the individual bee was in motion. Filled circles denote the undosed 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.

The higher food consumption of bumblebees is not attributable solely to body size because their mass was only 40% greater than that of the honey bees in the present study. Also, it cannot be attributed to a putative energetic cost of detoxifying imidacloprid because even undosed bumblebees consumed 6 times more syrup than undosed honey bees. Instead, it is speculated that bumblebees metabolised the syrup while maintaining relatively high body temperatures.[32] It is therefore hypothesised that their high energy requirement may predispose bumblebees to impacts from toxicants in nectar.

No evidence that imidacloprid accumulated persistently in either species was found. In the present experiment, adult honey bees cleared imidacloprid at the rate of ingestion, which is consistent with the previously reported biological half-life of about 4 h.[23] Even in bumblebees it was found that bodily residues equilibrated, and that the biological half-life of imidacloprid was only approximately 10 h. These 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 bumblebees were affected by dietary imidacloprid, they nevertheless cleared the toxicant from their bodies within 48 h 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 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 realised in bath-perfusion experiments on isolated neurones,[40] which 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 such as oilseed rape. The present 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.

Only imidacloprid was quantified, but it can be inferred 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 bumblebees, 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, the post-dose increase in the activity level of bumblebees in the pulsed exposure treatment is not attributed to the delayed production of a stimulatory derivative. Instead, two explanations are proposed. Firstly, withdrawal of a cholinergic agonist can increase the 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 the 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. It is therefore speculated that the heightened activity of bumblebees 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 bumblebees exhibit this behaviour.

Bumblebees are affected by dietary concentrations of imidacloprid that are far lower than those used in the present experiment,[7,28,44] which suggests that incomplete clearance of continuously ingested toxicant occurs irrespective of the level of dietary exposure. It is surprising that bumblebees are affected by rates of ingestion in the region of 1 ng day^{-1} of imidacloprid,[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 the present authors are the first to demonstrate that behavioural recovery from intoxication coincides with bodily clearance of the toxicant. This 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, the recovery of brood production by bumblebee (*B. terrestris*) queens has also been observed after a pulsed exposure to dietary imidacloprid of 14 days at dosages of up to 125 $\mu g\ L^{-1}$ (Laycock I and Cresswell JE, unpublished). It is therefore anticipated that recovery, in whole or in part, will generalise to various endpoints in this species.

The observation that bumblebees 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 bumblebees 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 was observed under laboratory conditions means that bumblebees recover their full performance under ecologically relevant conditions.

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

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