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Imidacloprid impairs performance on a model flower handling task in bumblebees (*Bombus impatiens*)

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Abstract

Bumblebees exposed to neonicotinoid pesticides collect less pollen on foraging trips. Exposed bumblebees are also slower to learn to handle flowers, which may account for reduced pollen collection. It is unclear, however, why neonicotinoid exposure slows learning to handle flowers. We investigated the effect of imidacloprid, a neonicotinoid pesticide, on bumblebee motor learning using a lab model of flower handling. Bumblebees learned to invert inside a narrow tube and lift a petal-shaped barrier to reach a reward chamber. Imidacloprid-exposed bumblebees showed a dose-dependent delay to solve the task, which resulted from reduced switching between behavioural strategies and a subsequent delay in use of the successful strategy. This effect was consistent in colonies exposed at 10 but not 2.6 ppb, suggesting a variable effect on individuals at lower doses. These results help to explain why exposed bumblebees are slow to learn to handle flowers and collect less pollen on foraging trips.

Keywords Bumblebee · Imidacloprid · Neonicotinoid · Flower handling · Learning · Cognition

Introduction

The ecological and economic importance of animal pollination—the majority of which is carried out by bees—is difficult to overstate (Rader et al. 2016). An estimated 87.5% of wild flowering plants and 37 of our 59 most important crops (responsible for 35% of all food production) rely on or benefit from animal pollination (Klein et al. 2007; Ollerton et al. 2011). Recent declines in bee populations have therefore received much attention (Aizen and Harder 2009; Goulson et al. 2008). Though the plight of the honey bee (*Apis mellifera* L.) has received most of this attention, non-*Apis* pollinators, the most widespread of which are bumblebees, also face problems (genus *Bombus*; Lundin et al. 2015). Bumblebees are experiencing particularly concerning declines in abundance and diversity, with an estimated one third of species in decline (Arbetman et al. 2017). Given the importance of bumblebees as temperate zone pollinators, it is crucial that these declines be addressed (Winfree et al. 2008; Garibaldi et al. 2013). Several interacting factors are responsible for bumblebee declines (Potts et al. 2010), including habitat loss and fragmentation (Kremen et al. 2007; Ricketts et al. 2008; Winfree et al. 2009), exposure to pathogens (Otti and Schmid-Hempel 2008; Graystock et al. 2014) and exposure to harmful pesticides (Godfray et al. 2015; Wood and Goulson 2017).

Neonicotinoid pesticides were introduced to the market in the 1990s and rapidly became the fastest-growing pesticide class in use (Jeschke et al. 2011). Neonicotinoids act by binding to nicotinic acetylcholine receptors in insects to lethally disrupt neural activity (Matsuda et al. 2001). They are typically applied as seed dressings, which integrate into growing plants and appear in nectar and pollen at levels ranging from <1 to 10 ppb (parts per billion; Sur and Stork 2003; Blacquière et al. 2012; Godfray et al. 2015). Further, because neonicotinoids are water-soluble and persist in the environment for long periods, they often integrate into wildflowers and appear in nectar and pollen at levels similar to those in treated crops (Botías et al. 2015; Morrissey et al. 2015; Long and Krupke 2016). Though the three most common neonicotinoids (imidacloprid, thiamethoxam and clothianidin) have been restricted for outdoor use in the EU (EU Press Release, 27 April 2018),

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neonicotinoids are still widely used in many countries, including the USA and China (Shao et al. 2013; Douglas and Tooker 2015) and thus present a non-negligible threat to global bumblebee populations.

Bumblebees may be more sensitive to neonicotinoids than honeybees are (Stoner and Eitzer 2012). Honeybee colonies are composed of thousands of individuals that overwinter as a colony unit and benefit substantially from social buffering. If a group of foragers from a large colony is affected by a stressor (e.g. a pesticide or pathogen), the colony is less likely to fail because there are many other individuals to replace them (Straub et al. 2015). Similarly, honeybee colonies are relatively quick to replace gynes (future queens) weakened by stressors because large colonies have the resources available to do so (Sandrock et al. 2014). Bumblebee colonies—which typically contain <500 individuals—benefit much less from social buffering. This is particularly the case during the colony initiation period when bumblebee queens emerge from overwintering to independently found new colonies; the first batch of workers takes ~4–5 weeks to develop (Alford 1975) and stressors that affect the queen or developing bees during this time can be devastating. Indeed, neonicotinoids have been shown to disrupt early bumblebee colony development (Baron et al. 2017; Wu-Smart and Spivak 2018) and growth throughout the season (Whitehorn et al. 2012; Arce et al. 2017). Furthermore, in field studies in which honeybee and bumblebee colonies are exposed equivalently to neonicotinoids, bumblebee colony growth is reduced more dramatically, partially because honeybees benefit more from social buferring (Rundlöf et al. 2015; Woodcock et al. 2017).

How might neonicotinoids be limiting bumblebee colony growth? One explanation is that exposed bumblebees collect fewer resources, which limits nutrient availability for developing offspring. This explanation has strong support, with many studies documenting pollen foraging deficits in exposed bumblebees (Feltham et al. 2014; Gill et al. 2012; Gill and Raine 2014; Stanley et al. 2016; Whitehorn et al. 2017). Pollen is the key protein resource for bumblebees, and critical for the nutrition of developing bees. How, then, might neonicotinoids reduce the foraging success of exposed workers? It may be that exposed bumblebees are less able to learn about and remember important features of the foraging environment. Bumblebees collect resources from a variety of flowers; to forage efficiently, it is therefore critical that they discriminate between flowers that differ on multiple dimensions (e.g. colour, scent, morphology) and pair floral features with food rewards (Chittka and Thomson 2001; Muth et al. 2015). Growing evidence suggests that neonicotinoid exposure disrupts the ability of bumblebees to effectively pair floral features with food rewards (Siviter et al. 2018; Muth et al. 2019). Most studies that test neonicotinoid-induced deficits in learning and memory have used the proboscis extension reflex (PER) protocol, where bees are removed from the colony, harnessed, conditioned to associate a stimulus (e.g. scent, colour) with a sucrose reward and later tested to see how well they remember the association. While deficits in PER learning are concerning, the PER protocol imperfectly reflects natural foraging scenarios in which bees must choose from a diverse range of floral resources (Muth et al. 2017). Studies that use the PER protocol are therefore insufficient to fully describe how neonicotinoid exposure may disrupt bumblebee learning and memory under environmentally relevant conditions. Recent work has begun to examine neonicotinoid effects on a wider range of learning and memory tasks. Using a free-flight multi-choice paradigm, Phelps et al. (2018) found neonicotinoid-induced deficits in colour-reward association learning and Muth et al. (2019) showed that exposure to neonicotinoids limits learning opportunities by reducing motivation.

It is not enough, however, for bumblebees to learn which flowers are rewarding—they must also be able to extract pollen and nectar from them. Flowers are morphologically diverse; while pollen and nectar are exposed and easily accessible in some, they are concealed in others, hidden in atypical locations or behind petals (Laverty 1980; Ronse De Craene 2010). To efficiently collect resources, bumblebees therefore must learn how to handle flowers of varying morphological complexity (Heinrich 1976, 1979; Laverty 1980; Laverty and Plowright 1988). Given the importance of flower handling to foraging, it is surprising that relatively few studies have tested whether neonicotinoid exposure disrupts flower handling in bumblebees. Impaired sonication behaviour (the extraction of pollen from flowers by buzzing) has been documented in exposed bumblebees (Switzer and Combes 2016; Whitehorn et al. 2017) and recent work by Stanley and Raine (2016) showed that exposed bumblebees take more trials to efficiently obtain pollen from novel, morphologically complex flowers. It is unclear, however, exactly which behavioural differences between exposed and unexposed bumblebees may contribute to this delay in learning to handle flowers.

In the present study, we examined the effects of imidacloprid, a common neonicotinoid pesticide, on flower handling in the North American bumblebee *Bombus impatiens*. We used a laboratory model of flower handling developed by Strang (2018) to accomplish this goal. Over each of 30 trials (10 trials per day across 3 days), bumblebees learned to manipulate a petal-shaped barrier to receive a sucrose reward. In addition to measuring latency to solve the task, we recorded in detail which behaviours bumblebees engaged in across trials, how long they engaged in them and how often they switched between them. This provided a rich exploratory behavioural comparison of exposed and unexposed bumblebees to

Table 1 Number of bumblebees tested from each colony

Exposure Level	Number of bumblebees tested		
	Colony 1	Colony 2	Colony 3
0 ppb	7	9	9
2.6 ppb	6	9	10
10 ppb	8	8	9

Nine different colonies were tested, three at each exposure level, for a total of 75 bumblebees tested. Twenty-five bumblebees were tested at each exposure level

supplement task performance data. We hypothesized that if imidacloprid negatively affects flower handling, exposed bumblebees would be slower to learn the correct petal-manipulation strategy and therefore take longer to solve the task across trials.

Methods

Subjects and colony maintenance

We sequentially tested bumblebees from nine *Bombus impatiens* colonies containing one queen and ~75 workers, all obtained from a commercial supplier (Biobest Canada Ltd, Leamington, ON). Each colony was kept on a 12-h light: 12-h dark schedule (onset 0700 h) and housed in a hive box (30.5(l) × 24(w) × 20(h) cm) connected to a flight cage (104(l) × 64(w) × 92(h) cm) by a 2 cm diameter Perspex® tube. Room illumination was provided by 32 W Philips PLUS T8 tubes (F32T8/TL841 PLUS ALTO HV, Philips Lighting Holding B.V.®). Flight cages contained four artificial flower patches with four artificial flowers each. Patches were rectangular sections of Styrofoam (20 × 32 cm) and artificial flowers were clear 1.7-ml micro-centrifuge tubes (Axygen Inc., Union City, CA) with 5 cm wide clear, plastic corollas. Bumblebees foraged *ad libitum* for 20% sucrose solution from artificial flower patches, which were replenished approximately hourly during testing sessions depending on foraging rate, using 5-ml syringes (VWR International Co.). The floor of the flight cage was covered in disposable surface protector (Whatman Benchkote®). Pollen collected by honeybees in North America was obtained from a commercial supplier (Biobest Canada Ltd, Leamington, ON); the granules were mixed with a small amount of 20% sucrose solution and this mixture was provided to bumblebees directly in the hive box. Each tested bumblebee was given a unique marking on the thorax with Posca paint markers (Mitsubishi Pencil Co.) for identification during testing; bumblebees were gently tagged while consuming sucrose solution in the testing apparatus and given a minimum of 1 h to habituate to the tag prior to testing. Twenty-five bumblebees were tested from each imidacloprid exposure level and a similar number of bumblebees were tested from each of the nine colonies (Median = 9; see Table 1 for more detail). Three colonies were exposed at each level of imidacloprid (0, 2.6 and 10 ppb); we therefore had three colony replicates per condition. Unexposed control colonies were housed in a separate room from the exposed colonies in the same research facility to prevent pesticide contamination of controls. The mean temperature of the facility was 21.6 °C (range 19.7–23.5 °C) and mean relative humidity was 43.7% (range 30–64%).

Preparation and provision of pesticide solution

Colonies were randomly assigned to chronic imidacloprid pesticide exposure in sucrose solution at 0, 2.6 or 10 ppb; thus, three colonies were exposed to imidacloprid at 2.6 ppb, three colonies were exposed at 10 ppb and three colonies were unexposed controls. All sucrose solution given to exposed colonies in artificial flowers and during testing contained that colony's imidacloprid treatment. This chronic method of exposure was chosen to simulate the experience of bumblebees repeatedly foraging on imidacloprid-treated crops.

Imidacloprid was obtained in dry powder form (Imidacloprid PESTANAL Fluka 37894, Sigma-Aldrich). Stock solutions of 1 ppm (part per million) imidacloprid were made by mixing 1 mg of imidacloprid in 1 l of distilled water using a vortex mixer. Fresh 300 ml 2.6 and 10 ppb imidacloprid sucrose solutions were made regularly by further diluting stock solution in fresh 20 and 40% sucrose solutions. Undosed 20 and 40% sucrose solutions for controls were also made in 300 ml batches. Sucrose concentration was verified using a refractometer. Solutions were stored away from sunlight in amber tinted glass bottles to prevent imidacloprid breakdown caused by UV light exposure (Soliman 2012).

In a previous study, we used liquid chromatography tandem-mass spectrometry to determine the concentration of imidacloprid-urea in the tissues of bumblebees given imidacloprid prepared by this dilution procedure and provided to bees by using the same feeding procedure as in the current study (Phelps et al. 2018). Bumblebees provided with 10 ppb solution had imidacloprid-urea concentrations ranging from 4.0–5.8 ng/g. In bumblebees provided with 2.6 ppb solution, however, tissue concentrations ranged from 0–0.12 ng/g.

Behavioural task

Testing apparatus

The testing apparatus consisted of a Perspex® reward chamber (6.4 cm^3) connected to the flight cage by a 2 cm

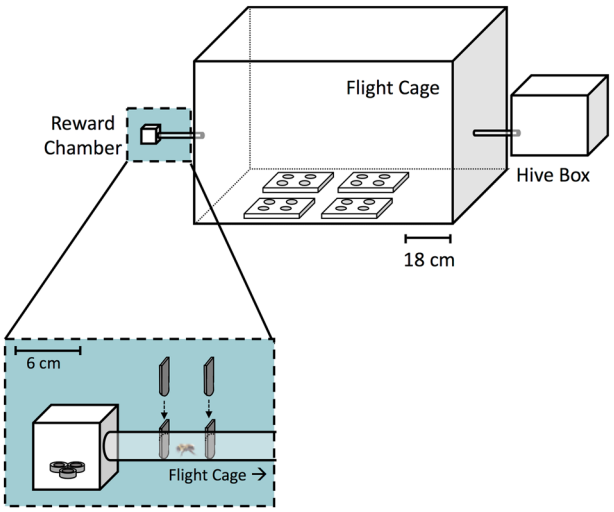

Fig. 1 The testing apparatus consisted of a 2 cm diameter Perspex® tube connected to the flight cage at one end and to a 6.4 cm^3 Perspex® reward chamber on the other. During trials, the plastic (rewarded) barrier was placed in the slot closest to the reward chamber and the metal (non-rewarded) barrier was placed in the slot closest to the flight cage. Bumblebees learned to invert and lift the plastic barrier to receive a sucrose reward. Microcentrifuge tube lids in the reward chamber contained 40% sucrose solution

diameter Perspex® tube (Fig. 1). The entrance to the tube was elevated 35 cm above the floor of the flight cage. An artificial flower was placed at the tube entrance and three 1.7-ml microcentrifuge tube lids were placed in the reward chamber; each of these was baited with 40% sucrose solution. The reward chamber had a hinged lid which was securely fastened by a magnetic closure. Six 3 mm diameter holes were drilled into the reward chamber for ventilation. There were two 1 cm deep slots 6.5 cm apart in the tube connecting the flight cage to the reward chamber. Plastic and metal barriers were placed through these slots during testing. Each barrier (25 × 13 mm) was rectangular but rounded at the bottom. The bottom of each barrier fit the shape of the tube, but not perfectly; there was a slight gap beneath each barrier through which a bumblebee could insert its forelegs to lift the barrier. The plastic (rewarded) barrier blocked access to the reward chamber but was light enough for bumblebees to lift. The metal (non-rewarded) barrier was too heavy to be lifted and blocked bumblebees from returning to the flight cage.

Pre-testing

Colonies were given a minimum of one week for imidacloprid exposure and habituation to foraging in the flight cage prior to testing. Bumblebees could move freely between the flight cage and the reward chamber during this time. The artificial flower at the tube entrance and three microcentrifuge tube lids inside the reward chamber were baited frequently, and bumblebees learned that these contained highly rewarding 40% sucrose solution. Bumblebees also had access to the four artificial flower patches containing artificial flowers with 20% sucrose solution. Only bumblebees foraging in the reward chamber, however, were marked and tested.

Testing procedure

We used a behavioural task designed by Strang (2018) to model the learning and execution of a motor pattern resembling flower handling. Bumblebees were required to manipulate the petal-shaped reward barrier to receive a sucrose reward. As with many actual flowers, this task had only one effective solution. The bee had to turn upside down in the tube and while inverted lift the barrier by grasping its bottom edge and pulling upwards. Bumblebees were individually tested for ten trials per day across three consecutive days for a total of 30 trials. The artificial flower by the tube entrance and the reward chamber were baited with 40% sucrose solution before testing to attract bumblebees and to facilitate the association between reward and reward chamber entry. Prior to testing, bumblebees were cleared from the reward chamber and sucrose solution in the artificial flower by the tube entrance was depleted.

During testing, a single marked bumblebee was allowed to enter the tube from the flight cage. The plastic barrier was inserted through the slot closest to the reward chamber and blocked the path to reward. Once the bumblebee was inside, the metal barrier was placed through the slot behind it to prevent it from leaving and other bumblebees from entering. Trials were video recorded using a GoPro camera (GoPro® Hero3+ Silver camera, GoPro Inc., San Mateo, CA). The trial ended when the bumblebee removed the plastic barrier or after 5 min had passed. Bumblebees that were successful in removing the plastic barrier entered the reward chamber to consume 40% sucrose solution to satiation. If a bumblebee failed to remove the plastic barrier, the barrier was removed by the experimenter and the bumblebee was allowed to consume 40% sucrose solution to satiation. Care was taken to ensure that unsuccessful bumblebees were not facing or interacting with the plastic barrier when it was removed. Once satiated, bumblebees were allowed to return to the colony. Sucrose solution was replenished between trials. The next trial began when the marked bumblebee returned to the testing apparatus and entered the tube. Because bumblebees self-initiated trials it was not possible to standardize inter-trial interval. Data collection occurred for ~20 days for each colony after the one-week habituation period, and this duration was similar across conditions.

Video analysis

Video analysis was conducted using Noldus Observer XT software (Noldus Information Technology, Wageningen, Netherlands) to quantify latency to remove the rewarded barrier and the proportion of time bumblebees spent using different behavioural strategies. The onset of a trial was defined as a bumblebee crossing the slot through which the metal barrier was placed. At that point, video scoring began and continued until the plastic barrier was removed or until 5 min had passed. Latency to remove the plastic barrier was recorded in seconds for each trial and a score of 300 s was given to bumblebees that failed to remove the plastic barrier. The two dominant strategies identified by Strang (2018) were quantified: inverting inside the tube to lift the barrier (a successful strategy) and pushing the barrier (an ineffective strategy). Inverting to lift was operationally defined as a bumblebee exerting pressure on the barrier with its head or forelegs while inverted at least 90° to the left or right. Pushing was operationally defined as a bumblebee exerting pressure on the barrier with its head or forelegs while upright. Inverting to lift and pushing were quantified separately at each barrier; thus, four behaviours were quantified overall: (1) inverting to lift the rewarded (plastic) barrier, (2) pushing the rewarded (plastic) barrier, (3) inverting to lift the non-rewarded (metal) barrier and (4) pushing the non-rewarded (metal) barrier. The proportion of time bumblebees engaged in these behaviours was obtained by dividing the time spent engaging in each by latency to remove the rewarded barrier. The rate at which bumblebees switched between behaviours was obtained by dividing the number of switches by latency to remove the rewarded barrier.

Measuring body size

Limited evidence shows that cognition is more severely affected by neonicotinoid exposure in larger bumblebees (Samuelson et al. 2016; but see Stanley et al. 2015). We therefore took body measurements to investigate this possibility. Head width (i.e. the distance between the lateral margin of the compound eyes) and thorax width (i.e. the distance between the lateral margin of the tegulae) were obtained from 60 bumblebees (19, 21 and 20 from colonies exposed at 0, 2.6 and 10 ppb respectively) using a caliper (EZ-Cal IP54 Digital Caliper, iGaging Precision Instruments®, San Clemente, California) with 0.01 mm precision. Measurements of head and thorax width were taken twice on separate days.

Statistical analysis

All statistical analyses were conducted using SPSS (Version 24, IBM Corp.) with an alpha level of 0.05. Greenhouse–Geisser corrections were used when the sphericity assumption of analysis of variance (ANOVA) was violated. Post hoc tests were conducted using the Bonferroni method when significant group differences were found.

Repeated measures ANOVAs were used to test for differences among imidacloprid exposure groups, colony differences, and the repeated effect of trial. Seven measures of behaviour were analyzed. Our key measure of task performance was latency for bumblebees to remove the rewarded barrier; latency was analyzed between imidacloprid groups as well as between colony replicates at each level of imidacloprid exposure. For ANOVAs comparing imidacloprid groups, a between-subjects factor of imidacloprid concentration (0, 2.6 and 10 ppb) and a within-subjects factor of trial (1–30) were used. For ANOVAs comparing colony replicates at each imidacloprid level, a between-subjects factor of colony (1, 2 and 3) and a within-subjects factor of trial (1–30) were used.

We analyzed the proportion of time bumblebees engaged in each of two key behaviours: (1) inverting to lift the rewarded barrier and (2) pushing the rewarded barrier. A separate ANOVA was conducted for each behaviour to compare the proportion of time bumblebees from different imidacloprid exposure groups engaged in each. A between-subjects factor of imidacloprid concentration (0, 2.6 and 10 ppb) and a within-subjects factor of trial (1–30) were used for each ANOVA. In addition to these individual behaviour analyses, we conducted a behavioural profile analysis to examine between-group differences more holistically. We included not only the proportion of time bumblebees inverted to lift and pushed the rewarded barrier, but also the proportion of time they inverted to lift and pushed the non-rewarded barrier (activity at the non-rewarded barrier was relatively infrequent, so we did not analyze it independently of behavioural profile). For the ANOVA comparing behavioural profile, a between-subjects factor of imidacloprid concentration (0, 2.6 and 10 ppb) and within-subjects factors of trial (1–30) and behaviour (inverting to lift the rewarded barrier, pushing the rewarded barrier, inverting to lift the non-rewarded barrier and pushing the non-rewarded barrier) were used.

We analyzed the rate at which bumblebees switched between inverting to lift and pushing the rewarded and non-rewarded barrier across trials. The rate was obtained for each trial by calculating the total number of behaviour switches and dividing the total by latency to remove the rewarded barrier. For this ANOVA, a between-subjects factor of imidacloprid concentration (0, 2.6 and 10 ppb) and a within-subjects factor of trial (1–30) were used.

A Pearson correlation was used to examine the relationship between head and thorax measurements taken on days 1 and 2. Day 1 and 2 measurements were highly

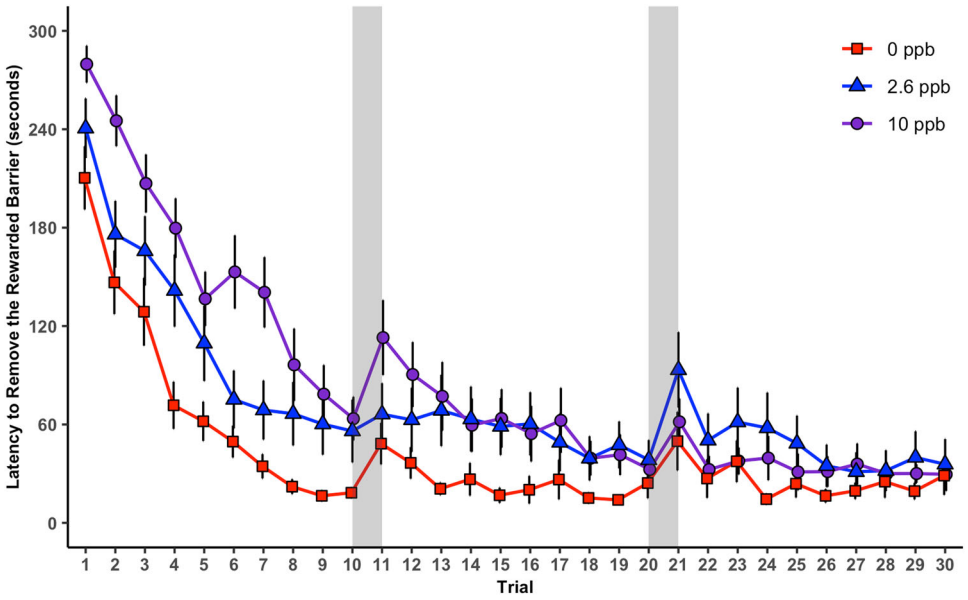

Fig. 2 Latency to remove the rewarded barrier decreased over trials. Imidacloprid-exposed bumblebees showed a greater latency to remove the rewarded barrier that was dose-dependent, but all bees performed similarly by trial 30. Vertical bars represent overnight retention intervals between days 1 and 2 and between days 2 and 3. Bumblebees were slower to remove the rewarded barrier following the second overnight retention interval, but not the first. Error bars equal ±1 standard error of the mean. See text for statistical results

correlated for head width ($r(58) = 0.98$, $p < 0.001$) and thorax width ($r(58) = 0.99$, $p < 0.001$), so the mean of day 1 and 2 measurements was calculated. A Pearson correlation was then used to examine the relationship between head and thorax width. These were also highly correlated ($r(58) = 0.94$, $p < 0.001$), and therefore only thorax width was used subsequently. Pearson correlations were used to examine the relationship between thorax width and composite latency to remove the rewarded barrier for bumblebees in each condition and overall (i.e. with all groups combined). A one-way ANOVA with the between-subjects factor of imidacloprid concentration (0, 2.6 and 10 ppb) was used to confirm there were no differences in bumblebee thorax width between conditions.

Results

Latency to remove the rewarded barrier

Latency between imidacloprid exposure groups

There was a significant imidacloprid concentration by trial interaction, ($F(22.23, 800.22) = 2.408$, $p < 0.001$, $\eta_p^2 = 0.06$). Bumblebees from all groups became faster to remove the rewarded barrier across trials, but those exposed to imidacloprid were initially much slower to solve the task (Fig. 2). A main effect of imidacloprid concentration ($F(2, 72) = 5.972$, $p = 0.004$, $\eta_p^2 = 0.14$) followed by post hoc tests revealed that unexposed bumblebees were faster to remove the rewarded barrier than those exposed at 10 ppb ($p = 0.004$) but not significantly faster than those exposed at 2.6 ppb ($p = 0.058$). Bumblebees exposed at 2.6 and 10 ppb did not significantly differ ($p = 1.000$). A main effect of trial ($F(11.11, 800.22) = 54.056$, $p < 0.001$, $\eta_p^2 = 0.43$) showed that bumblebees became much faster to remove the rewarded barrier over time. Post hoc tests revealed no difference in latency to remove the rewarded barrier between trial 10 and 11 (following overnight retention interval one) but bumblebees were slower to remove the rewarded barrier following overnight retention interval two between trial 20 and 21 ($p = 0.033$).

The previous analysis revealed that exposed bumblebees are initially slower to solve the task on a group level, but this effect requires further clarification because trials were capped at 300 s. There are three possibilities that may explain these results: (1) exposed bumblebees successfully solved the task in <300 s as frequently as unexposed bumblebees but took longer on average, (2) exposed bumblebees solved the task less frequently than unexposed bumblebees, but those that did were as fast as the unexposed bumblebees, or (3) exposed bumblebees solved the task less frequently and more slowly than unexposed bumblebees. We report the number of bumblebees from each condition that solved the task in <300 s and the mean latency for these bumblebees to solve the task across trials in Table 2. The information in Table 2 provides support for the third possibility; exposed bumblebees, particularly those exposed at 10 ppb, solve the task in <300 s less frequently during early trials, and those that did solve it solved it more slowly on average than unexposed bumblebees.

Latency between colony replicates

Unexposed colonies There was a significant main effect of trial ($F(7.45, 164.00) = 20.176$, $p < 0.001$, $\eta_p^2 = 0.48$),

Table 2 Number of bumblebees that solved the task across trials, with percentage out of 25 shown in parentheses

Trial	0 ppb ($n = 25$)	2.6 ppb ($n = 25$)	10 ppb ($n = 25$)
1	15 (60%), 150.60	10 (40%), 151.53	4 (16%), 172.97
2	20 (80%), 108.28	19 (76%), 136.76	14 (56%), 202.10
3	22 (88%), 105.37	17 (68%), 102.71	16 (64%), 154.56
4	24 (96%), 62.17	18 (72%), 80.02	18 (72%), 132.93
5	25 (100%), 61.94	21 (84%), 73.21	22 (88%), 114.32
6	25 (100%), 49.55	24 (96%), 65.88	17 (68%), 83.79
7	25 (100%), 34.49	23 (92%), 48.67	19 (76%), 90.23
8	25 (100%), 22.09	22 (88%), 34.71	20 (80%), 45.45
9	25 (100%), 16.49	23 (92%), 39.37	23 (92%), 59.20
10	25 (100%), 18.36	22 (88%), 22.71	24 (96%), 53.68
11	25 (100%), 48.31	22 (88%), 34.38	21 (84%), 77.34
12	25 (100%), 36.55	22 (88%), 30.45	22 (88%), 61.86
13	25 (100%), 20.79	21 (84%), 24.44	21 (84%), 34.71
14	25 (100%), 26.59	22 (88%), 31.02	24 (96%), 49.53
15	25 (100%), 16.83	23 (92%), 37.80	23 (92%), 43.10
16	25 (100%), 20.19	22 (88%), 27.71	24 (96%), 44.13
17	24 (96%), 15.02	23 (92%), 27.23	24 (96%), 52.56
18	25 (100%), 15.17	24 (96%), 28.46	25 (100%), 39.41
19	25 (100%), 14.02	25 (100%), 47.52	25 (100%), 41.51
20	25 (100%), 24.27	24 (96%), 27.48	25 (100%), 32.58
21	23 (92%), 28.06	20 (80%), 41.60	25 (100%), 61.62
22	24 (96%), 15.65	23 (92%), 28.61	25 (100%), 32.51
23	25 (100%), 37.52	22 (88%), 28.93	25 (100%), 37.73
24	25 (100%), 14.48	22 (88%), 24.86	24 (96%), 28.66
25	25 (100%), 23.86	24 (96%), 37.96	25 (100%), 31.04
26	25 (100%), 16.49	24 (96%), 23.75	25 (100%), 31.25
27	25 (100%), 19.53	24 (96%), 19.95	24 (96%), 24.83
28	25 (100%), 25.13	24 (96%), 20.64	25 (100%), 30.01
29	25 (100%), 19.36	23 (92%), 17.23	25 (100%), 30.06
30	24 (96%), 17.52	24 (96%), 24.72	25 (100%), 29.55

Mean latency is shown in seconds for bumblebees that solved the task in <300 s

indicating that bumblebees became faster to remove the rewarded barrier across trials overall. There was no significant main effect of colony ($F(2, 22) = 0.741$, $p = 0.488$, $\eta_p^2 = 0.06$) or trial by colony interaction ($F(14.91, 164.00) = 0.849$, $p = 0.621$, $\eta_p^2 = 0.07$), however, indicating that unexposed colonies performed similarly (Fig. 3).

Colonies exposed at 2.6 ppb There was a significant main effect of trial ($F(7.05, 155.13) = 11.064$, $p < 0.001$, $\eta_p^2 = 0.33$), indicating that bumblebees became faster to remove the rewarded barrier across trials overall. There was also a significant main effect of colony ($F(2, 22) = 8.278$, $p = 0.002$, $\eta_p^2 = 0.43$), indicating that colonies exposed at 2.6 performed differently (Fig. 3). Post hoc tests revealed that bumblebees from colony one and three performed similarly ($p = 1.000$), but that bumblebees from colony two were slower to remove the rewarded barrier than those from colony one ($p = 0.006$) and three ($p = 0.007$). There was no significant colony by trial interaction ($F(14.10, 155.13) = 0.982$, $p = 0.475$, $\eta_p^2 = 0.08$).

Colonies exposed at 10 ppb There was a significant main effect of trial ($F(7.17, 157.73) = 28.180$, $p < 0.001$, $\eta_p^2 = 0.56$), indicating that bumblebees became faster to remove the rewarded barrier across trials overall. There was no significant main effect of colony ($F(2, 22) = 0.813$, $p = 0.457$, $\eta_p^2 = 0.07$) or colony by trial interaction ($F(14.40, 157.73) = 1.108$, $p = 0.354$, $\eta_p^2 = 0.07$), however, indicating that colonies exposed at 10 ppb performed similarly (Fig. 3).

Use of behavioural strategies

Behavioural profile

There was a significant imidacloprid concentration by trial by behaviour interaction, indicating that bumblebees exposed to different concentrations of imidacloprid differed behaviourally over time ($F(34.63, 1246.59) = 1.879$, $p = 0.002$, $\eta_p^2 = 0.05$). Figure 4 shows that imidacloprid-exposed bumblebees were slower to shift from using the incorrect behavioural strategy (pushing) to using the correct strategy (inverting to lift). A main effect of imidacloprid concentration ($F(2, 72) = 6.016$, $p = 0.004$, $\eta_p^2 = 0.14$) followed by post hoc tests revealed that unexposed bumblebees differed significantly in behavioural profile from those exposed at 10 ($p = 0.008$) but not 2.6 ppb ($p = 1.000$), and that bumblebees exposed at 2.6 also differed from those exposed at 10 ppb ($p = 0.016$). That is, while bumblebees exposed at 0 and 2.6 ppb performed similarly, those exposed at 10 ppb were more likely to favour the pushing strategy. Despite this, a significant trial by behaviour interaction ($F(17.31, 1246.59) = 34.884$, $p < 0.001$, $\eta_p^2 = 0.33$) shows that regardless of imidacloprid exposure, bumblebees abandoned the non-rewarded barrier and shifted from pushing the rewarded barrier to inverting to lift it.

Inverting to lift the rewarded barrier

There was a significant trial by imidacloprid concentration interaction ($F(26.30, 946.73) = 2.776$, $p < 0.001$, $\eta_p^2 = 0.07$). Bumblebees increased the proportion of time spent inverting to lift the rewarded barrier across trials, but this increase was slower for those exposed to imidacloprid. These data (also shown in Fig. 4) are re-plotted in Fig. 5 to make comparisons between imidacloprid treatment groups clearer. There was a significant main effect of trial ($F(13.15, 946.73) = 50.840$, $p < 0.001$, $\eta_p^2 = 0.41$), indicating that

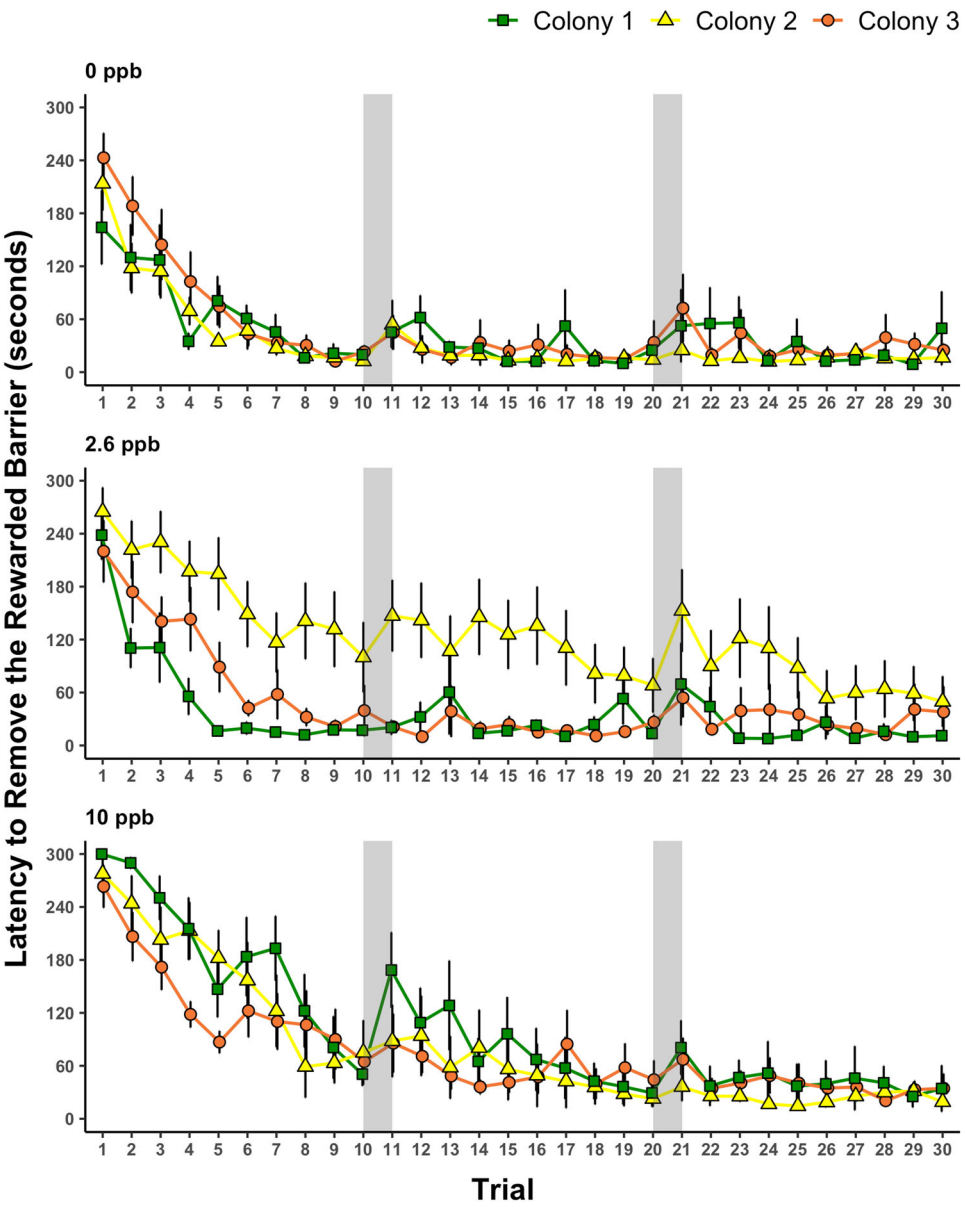

Fig. 3 Latency to remove the rewarded barrier. Bumblebees from unexposed colonies one, two and three performed similarly. Bumblebees from 2.6 ppb imidacloprid-exposed colony two were slower to remove the barrier than those from colony one and three. Bumblebees from 10 ppb imidacloprid-exposed colonies one, two and three performed similarly. Vertical bars represent overnight retention intervals between days 1 and 2 and between days 2 and 3. Error bars equal ±1 standard error of the mean. See text for statistical results

bumblebees increased in the proportion of time spent inverting to lift the rewarded barrier across trials; post hoc tests revealed that bumblebees spent a lower proportion of time inverting to lift the rewarded barrier on trial 11 than on trial 10 (following overnight retention interval one; $p = 0.012$) and a lower proportion following overnight retention interval two ($p < 0.001$). There was no significant main effect of imidacloprid concentration independent of trial ($F(2, 72) = 1.334$, $p = 0.270$, $\eta_p^2 = 0.04$).

Pushing the rewarded barrier

A significant main effect of trial was found ($F(12.40, 893.12) = 19.379$, $p < 0.001$, $\eta_p^2 = 0.21$), indicating that bumblebees differed in the proportion of time they spent pushing the rewarded barrier across trials. Bumblebees spent an increasing proportion of time pushing the rewarded barrier from trial one to trial six, and a generally decreasing proportion of time across remaining trials. These data (also shown in Fig. 4) are re-plotted in Fig. 6 to make comparisons between imidacloprid treatment groups clearer. A main effect of imidacloprid concentration was not significant ($F(2, 72) = 2.687$, $p = 0.075$, $\eta_p^2 = 0.07$) and there was no significant imidacloprid concentration by trial interaction ($F(24.81, 893.12) = 1.311$, $p = 0.142$, $\eta_p^2 = 0.03$).

Overall proportion of time pushing or inverting to lift

A significant main effect of imidacloprid concentration ($F(2, 72) = 6.016$, $p = 0.004$, $\eta_p^2 = 0.14$) followed by

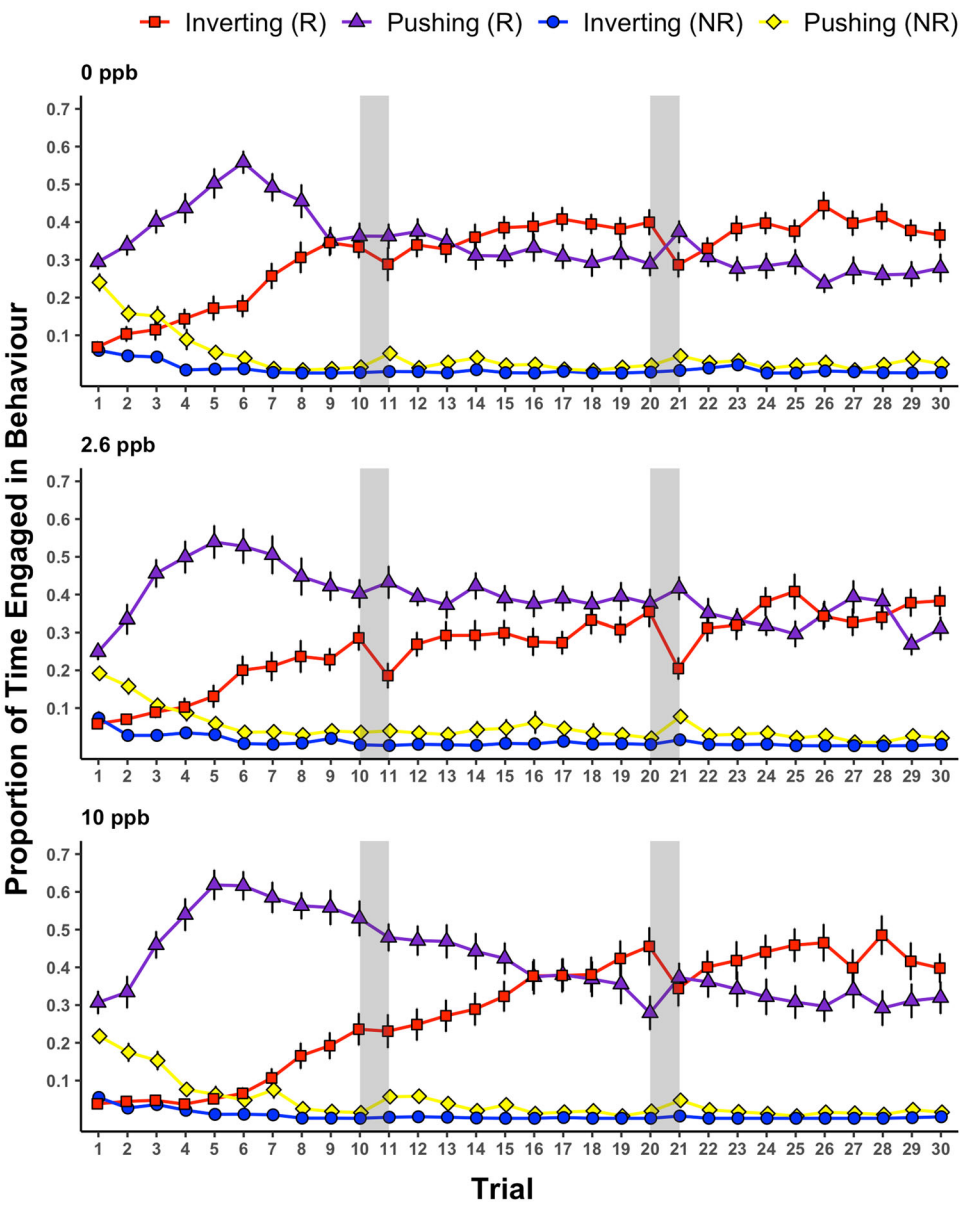

Fig. 4 Proportion of time engaging in four behaviours: inverting to lift the rewarded (R) and non-rewarded (NR) barriers and pushing the rewarded and non-rewarded barriers. Unexposed bumblebees and bumblebees exposed at 2.6 ppb behaved similarly, but those exposed at 10 ppb took longer to shift to inverting to lift the plastic barrier. Bumblebees in all groups rapidly abandoned the non-rewarded barrier. Vertical bars represent overnight retention intervals between days 1 and 2 and between days 2 and 3. Error bars equal ±1 standard error of the mean. See text for statistical results

post hoc tests revealed that bumblebees exposed to imidacloprid at 10 ppb spent a greater proportion of time pushing or inverting to lift than unexposed bumblebees ($p = 0.008$) and those exposed at 2.6 ppb ($p = 0.016$) overall, but that unexposed bumblebees and those exposed at 2.6 ppb did not differ ($p = 1.000$; Fig. 7). There was a significant main effect of trial ($F(16.38, 1179.16) = 6.898$, $p < 0.001$, $\eta_p^2 = 0.09$), indicating that bumblebees increased in proportion of time pushing or inverting to lift on day 1 and declined slightly thereafter, though bumblebees exposed at 10 ppb did not decline. An interaction between imidacloprid concentration and trial fell short of significance ($F(32.75, 1179.16) = 1.437$, $p = 0.053$, $\eta_p^2 = 0.04$).

Behaviour switching

A significant main effect of imidacloprid concentration ($F(2, 72) = 6.859$, $p = 0.002$, $\eta_p^2 = 0.16$) followed by post hoc tests revealed that unexposed bumblebees switched between behaviours at a higher rate than bumblebees exposed at 2.6 ($p = 0.006$) and 10 ppb ($p = 0.006$), but that bumblebees exposed at 2.6 and 10 ppb did not differ ($p = 1.000$; Fig. 8). There was also a significant main effect of trial ($F(17.91, 1289.90) = 2.334$, $p = 0.001$, $\eta_p^2 = 0.03$); bumblebees showed a high rate of switching on trial one that dipped slightly during day 1 and stabilized thereafter. There was no significant interaction between trial and imidacloprid concentration ($F(35.83, 1289.90) = 1.231$, $p = 0.166$, $\eta_p^2 = 0.03$).

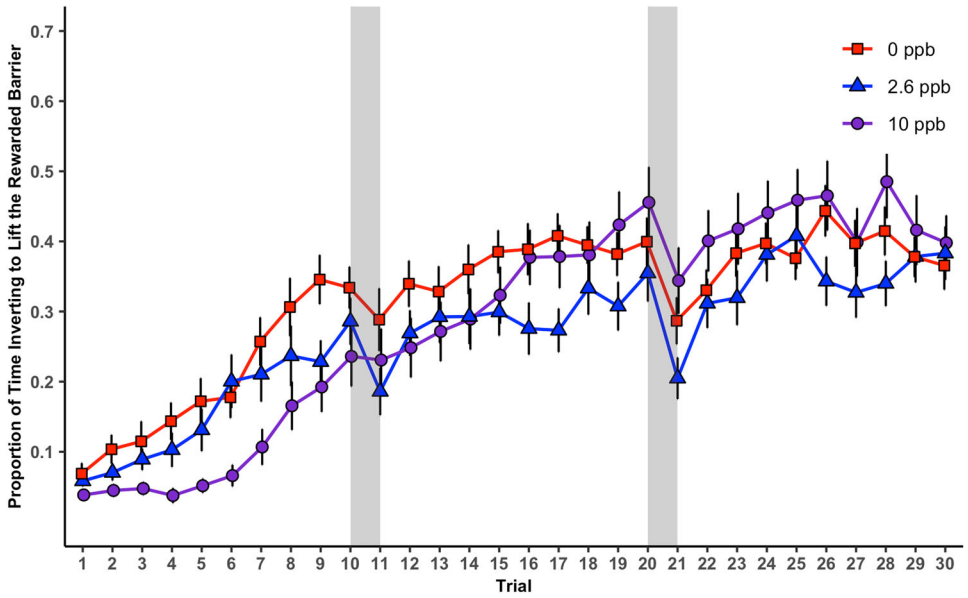

Fig. 5 Proportion of time inverting to lift the rewarded barrier. Bumblebees in all conditions spent a greater proportion of time inverting to lift the rewarded barrier across trials but this increase was slowest for bumblebees exposed at 10 ppb. Vertical bars represent overnight retention intervals between days 1 and 2 and between days 2 and 3. Bumblebees showed reductions in inverting to lift the rewarded barrier after both overnight retention intervals. Error bars equal ±1 standard error of the mean. See text for statistical results

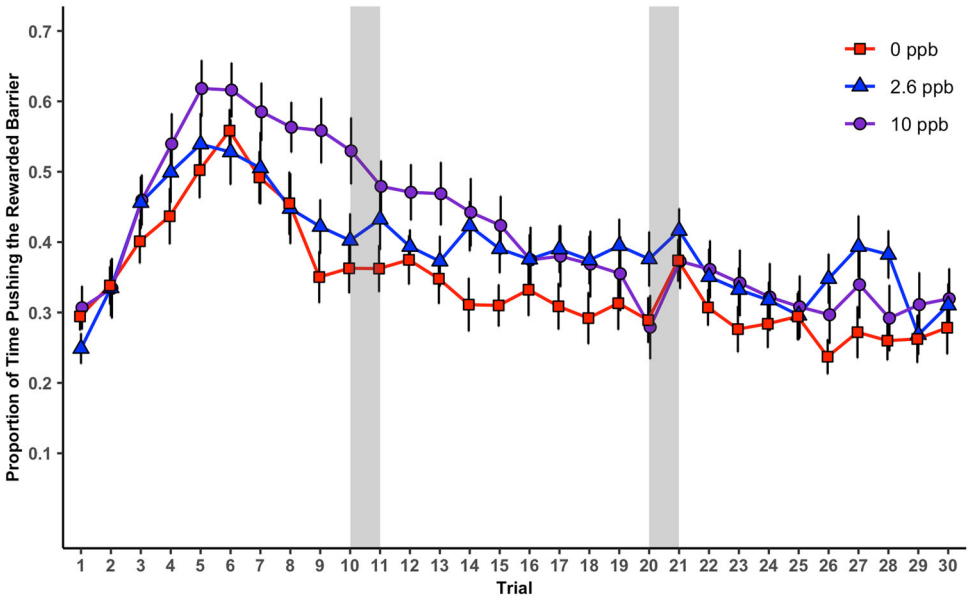

Fig. 6 Proportion of time pushing the rewarded barrier. Bumblebees increased in proportion of time pushing the rewarded barrier to trial six and decreased thereafter. Bumblebees exposed at 10 ppb experienced the greatest increase up to trial six and decreased at the slowest rate. Vertical bars represent overnight retention intervals between days 1 and 2 and between days 2 and 3. Error bars equal ±1 standard error of the mean. See text for statistical results

Body size

Bumblebees did not differ significantly in thorax width by condition ($F(2, 59) = 2.004$, $p = 0.144$, $\eta_p^2 = 0.07$). Thorax width and latency to remove the rewarded barrier were not related in unexposed bumblebees ($r(17) = -0.31$, $p = 0.200$), those exposed at 2.6 ppb ($r(19) = -0.26$, $p = 0.247$) or those exposed at 10 ppb ($r(18) = 0.05$, $p = 0.834$).

Discussion

There is strong evidence from field and semi-field studies that neonicotinoid exposure inhibits pollen collection (Feltham et al. 2014; Gill and Raine 2014; Whitehorn et al. 2017) and therefore colony growth (Whitehorn et al. 2012; Rundlöf et al. 2015; Arce et al. 2017; Woodcock et al. 2017) in bumblebees. To collect pollen efficiently, bumblebees must learn to discriminate between floral resources in the environment and extract resources from them. While much work has examined how neonicotinoids affect colour- and scent-reward association learning in bumblebees (Siviter et al. 2018; Phelps et al. 2018; Muth et al. 2019), their effects on floral resource extraction is less clear. We examined the effect of imidacloprid, a common neonicotinoid, on bumblebee flower handling using a novel model which required that bumblebees learn to manipulate a petal-shaped barrier over each of 30 trials to receive a food

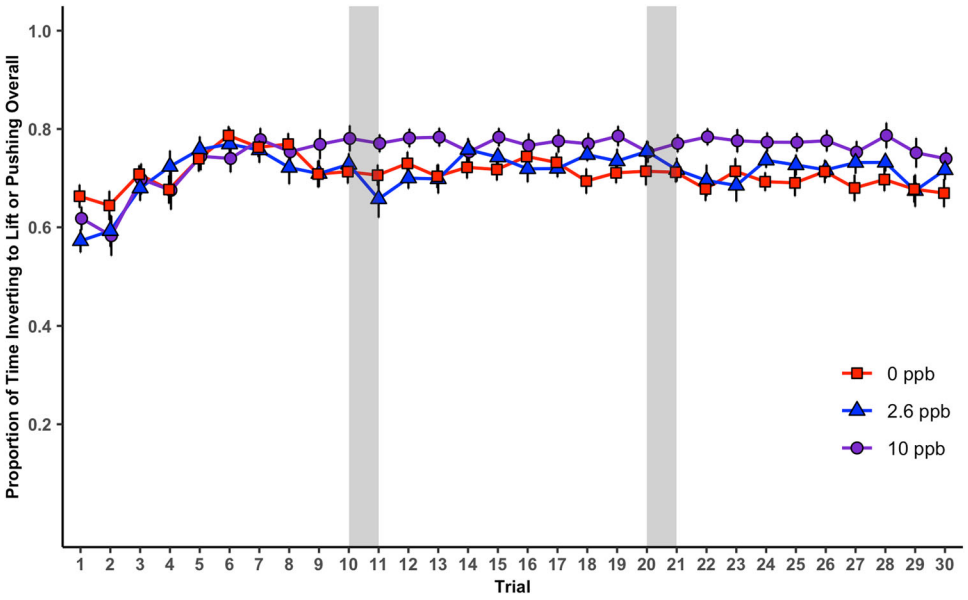

Fig. 7 Overall proportion of time inverting to lift or pushing either barrier. Bumblebees increased overall in proportion of time inverting to lift or pushing on day 1 and remained stable thereafter, with bumblebees exposed at 10 ppb spending a slightly greater proportion of time inverting to lift or pushing. Vertical bars represent overnight retention intervals between days 1 and 2 and between days 2 and 3. Error bars equal ±1 standard error of the mean. See text for statistical results

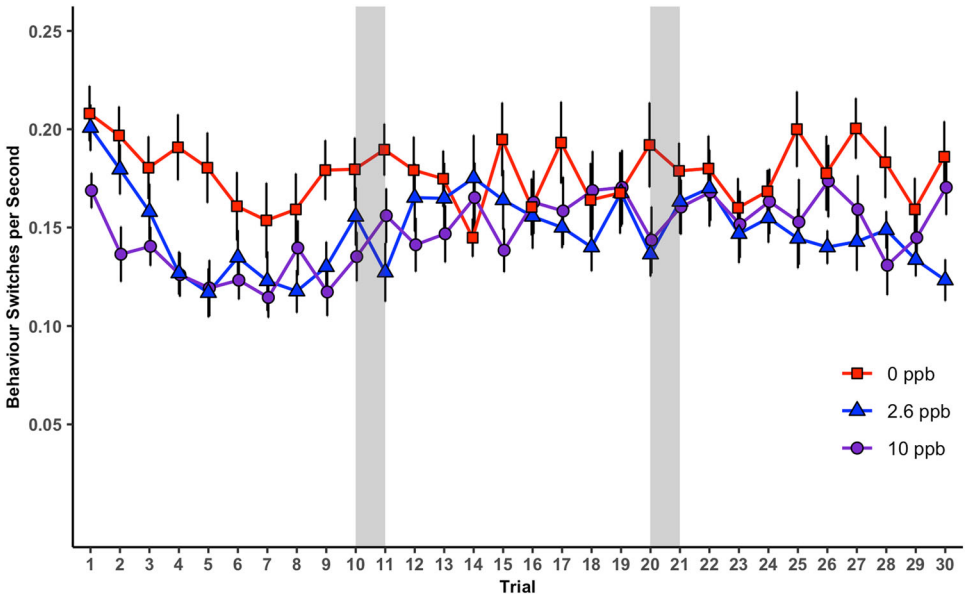

Fig. 8 Number of behaviour switches per second. Unexposed bumblebees switched between behaviours at a higher rate than did bumblebees exposed at 2.6 or 10 ppb during early trials. Vertical bars represent overnight retention intervals between days 1 and 2 and between days 2 and 3. Errors bars equal ±1 standard error of the mean. See text for statistical results

reward (Strang 2018). We found that imidacloprid exposure reduced the speed at which bumblebees were initially able to solve the task: fewer exposed bumblebees were able to solve the task before the 5-min cap during early trials, and those that did solve it were slower to do so than unexposed bumblebees. This impairment was dose-dependent across trials on the first day of testing, with bumblebees exposed at 10 ppb solving the task least frequently and most slowly. Despite this strong dose-dependent effect on day 1, imidacloprid-induced deficits diminished over time; the effect was less pronounced on day 2 and bumblebees performed similarly regardless of exposure by the end of testing on day 3. These results are consistent with those of Stanley and Raine (2016), which showed that bumblebees chronically exposed to the neonicotinoid thiamethoxam at 10 ppb took more trials to learn to handle morphologically complex flowers. We also found some evidence for overnight forgetting on the task, though not as a function of imidacloprid exposure; bumblebees were slower to solve the task following the second overnight retention interval but not the first. Consistent with Strang (2018), bumblebees solved the task faster across trials. This increased efficiency following experience parallels observations of bumblebees learning to forage on real flowers (Heinrich 1979; Laverty 1980; Peat and Goulson 2005; Raine and Chittka 2007).

We performed a detailed analysis of how imidacloprid affects behaviour during the learning process. Bumblebees used two dominant behavioural strategies to solve the task:

inverting in the tube to lift the rewarded barrier and pushing the rewarded barrier. Only inverting, however, was successful, which is consistent with Strang (2018). Bumblebees favoured the pushing strategy during early trials, likely because of its relative simplicity, but shifted to using the inverting to lift strategy after learning it was most effective. Bumblebees exposed to imidacloprid at 10 ppb were slower to shift from pushing to inverting to lift; they showed the strongest preference for pushing during early trials and spent less time inverting to lift on day 1 than did bumblebees exposed at 0 or 2.6 ppb. While unexposed bumblebees and those exposed at 2.6 ppb split time about evenly between pushing and inverting to lift by the end of day 1, bumblebees exposed at 10 ppb showed a preference for pushing until midway through day 2. Unexposed bumblebees showed a slightly stronger preference for inverting to lift than those exposed at 2.6 ppb across early trials, however, this difference was not statistically significant. This delay in exposed bumblebees to acquire the correct strategy is consistent with their delay to solve the task and likely explains why exposed bumblebees were slower to solve the task across trials.

Why were exposed bumblebees slower to use the successful behavioural strategy? The reason is not that exposed bumblebees were less active. Bumblebees in all conditions spent a similar amount of time interacting with the two barriers. Further, it is not the case that exposed bumblebees spent more time interacting with the unrewarded metal barrier. Bumblebees in all conditions decreased equally in the proportion of time spent interacting with the unrewarded barrier and rarely did so after day 1. Exposed bumblebees appeared slower to use the successful strategy because they were behaviourally less exploratory. They switched between behaviours at a slower rate than did unexposed bumblebees—particularly during day 1—and were more likely to persist using one strategy for long periods. Past work has shown that naïve bumblebees make frequent errors foraging on novel flowers but quickly improve through trial and error (Laverty 1980), learning associatively which behavioural strategies result in successful manipulation of flowers and subsequent reward. It seems reasonable, therefore, that a delayed process of trial and error limited the opportunities for associative learning and delayed acquisition of the successful strategy. Reduced exploration demonstrated here is consistent with past work by Phelps et al. (2018), which showed that imidacloprid-exposed bumblebees were slower to learn which flowers are rewarding because of reduced flower sampling.

Why did imidacloprid-exposed bumblebees show a reduction in behaviour switching but not in overall activity? Though perhaps initially surprising, past studies have yielded similar results. Williamson et al. (2014) found that honeybees exposed to thiamethoxam at field-realistic doses were not less active but suffered coordination deficits such as impaired performance of the righting reflex. Similarly, Whitehorn et al. (2017) showed that thiamethoxam-exposed bumblebees could not fine-tune buzz pollination to maximize pollen collection. While neonicotinoids at sufficiently high doses unsurprisingly cause major activity reduction and paralysis (Godfray et al. 2015; Moffat et al. 2016; Switzer and Combes 2016; Williamson et al. 2014), effects at field-realistic levels are more subtle. The distribution of neonicotinoid-sensitive receptors in critical motor control areas of the bee nervous system (the neuromuscular junctions and the ganglia of the ventral nerve cord) is less well understood than the distribution in learning and memory areas (the mushroom bodies and antennal lobes; Barbara et al. 2008; Deglise et al. 2002; Palmer et al. 2013). It was recently found, however, that honeybees exposed to imidacloprid at 10 ppb showed significant down-regulation of muscle-related genes (Wu et al. 2017). This down-regulation, in addition to the blockage of nicotinic acetylcholine receptors, may underlie the subtle motor deficits observed here and elsewhere.

While subtle imidacloprid-induced motor deficits were probably responsible for reduced behaviour switching, they may have acted in concert with associative learning deficits to cause delayed use of the successful motor strategy in bumblebees exposed at 10 ppb. Many studies have shown associative learning deficits in bees exposed to neonicotinoids at sub-lethal levels (Decourtye et al. 2004a, 2004b; Han et al. 2010; Phelps et al. 2018; Stanley et al. 2015; Williamson and Wright 2013). Exposed bees often require more learning trials to form associations between stimuli. It may be the case, therefore, that exposed bumblebees not only gained experience with the successful motor strategy at a slower rate, but also required more experience using the strategy to form a strong association between it and the food reward.

Bumblebees were of similar size across conditions and task performance did not vary as a function of body size. This finding conflicts with past work showing that larger bumblebees are typically faster to learn across varied contexts (Laverty 1994a; Stanley et al. 2015; Worden et al. 2009) and that memory in larger bees is more severely affected by neonicotinoid exposure (Samuelson et al. 2016; but see Stanley et al. 2015). Bumblebees ranged widely in thorax width (3.62–5.41 mm) but no size-related differences in behaviour were detected.

Bumblebee colonies often differ in the degree to which they are affected by pesticides (Phelps et al. 2018; Whitehorn et al. 2017). While bumblebees from colonies exposed at 0 and 10 ppb performed similarly across replicates, bumblebees from one colony exposed at 2.6 ppb performed significantly worse than others. Differences between bumblebees exposed at 0 and 2.6 ppb appear largely driven by

the poor performance of bumblebees from this 2.6 ppb colony and should thus be interpreted with caution. Consistent performance in colonies exposed at 0 and 10 ppb, however, does point to a robust effect of imidacloprid on motor learning at the level of 10 ppb. The colony variation problem is sometimes avoided by removing bumblebees (individually or in small groups) from a single colony and testing them at each exposure level (Piiroinen and Goulson 2016; Piiroinen et al. 2016; Stanley et al. 2015). Removing bumblebees from a natural colony context, however, reduces ecological validity and is not possible in studies of chronic pesticide exposure where bumblebees must return to the colony to deposit sucrose between trials.

One limitation of our study is that we did not examine individual and colony foraging rates, which would have allowed us to examine the effects of imidacloprid on motivation more meaningfully. Neonicotinoids have been shown to inhibit foraging in bumblebees, both through antifeedant effects and sub-lethal effects on activity (Laycock et al. 2012; Mommaerts et al. 2010; Thompson et al. 2014; Muth and Leonard 2019). Two laboratory studies have shown that neonicotinoid-exposed bumblebees allowed to forage on an array of artificial flowers are less exploratory, visiting fewer flowers and therefore limiting their potential to learn about novel food sources (Lämsä et al. 2018; Phelps et al. 2018). While we did not measure foraging rates, our finding that exposed bumblebees were less behaviourally exploratory while engaged in the task during early trials parallels the results of these past studies, suggesting that neonicotinoids may have subtle effects on foraging motivation.

While the flower handling model used here was designed to resemble a morphologically complex flower, many flowers important to bees are morphologically simple and thus easier to handle (Laverty 1980, 1994a; Strang 2018). It would be valuable to test whether neonicotinoid exposure impacts handling of morphologically simple flowers. It may be the case that exposed bumblebees can learn to handle simple flowers as effectively as unexposed bees but that subtle motor deficits reduce the efficiency with which they handle flowers over the tens to hundreds of flower visits comprising a typical foraging bout in the wild (Brunet 2009; Raine and Chittka 2007). Importantly, individuals often visit more than one flower species during foraging bouts (Chittka et al. 1997; Heinrich 1979) and must learn several associations between floral features and reward value simultaneously (Muth et al. 2015). Bumblebees switch between handling different flower species with an impressively low interference cost (Laverty 1994b; Strang 2018; Woodward and Laverty 1992). It would be valuable to test whether neonicotinoid exposure exacerbates memory interference in bumblebees learning to handle multiple flowers.

In summary, imidacloprid exposure caused a dose-dependent delay in performance on a motor task analogous to flower handling. Reduced performance in exposed bumblebees resulted from delayed acquisition of the successful motor strategy, which itself likely resulted from reduced behaviour switching during early trials. This effect occurred consistently in colonies exposed at 10 ppb. At 2.6 ppb, however, the effect was largely driven by bumblebees from one disproportionately affected colony. Results suggest, therefore, that imidacloprid exposure at 10 ppb causes clear deficits in motor learning but that exposure at lower doses in the field-realistic range may have variable effects across colonies. It is critical that bumblebees learn to handle flowers to efficiently collect pollen. These results help to explain why bumblebees exposed to neonicotinoids collect less pollen while foraging in the field (Feltham et al. 2014; Gill et al. 2012; Gill and Raine 2014; Stanley et al. 2016), which in turn helps to explain neonicotinoid-induced reducations in colony growth (Rundlöf et al. 2015; Whitehorn et al. 2012; Woodcock et al. 2017).

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Compliance with ethical standards

Conflict of interest The authors declare that they have 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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