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Imidacloprid slows the development of preference for rewarding food sources in bumblebees (*Bombus impatiens*)

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Abstract Bee pollination is economically and ecologically vital and recent declines in bee populations are therefore a concern. One possible cause of bee declines is pesticide use. Bumblebees exposed to imidacloprid, a neonicotinoid pesticide, have been shown to be less efficient foragers and collect less pollen on foraging trips than unexposed bees. We investigated whether bumblebees (*Bombus impatiens*) chronically exposed to imidacloprid at field-realistic levels of 2.6 and 10 ppb showed learning deficits that could affect foraging. Bumblebees were tested for their ability to associate flower colour with reward value in a simulated foraging environment. Bumblebees completed 10 foraging trips in which they collected sucrose solution from artificial flowers that varied in sucrose concentration. The reward quality of each artificial flower was predicted by corolla colour. Unexposed bumblebees acquired a preference for feeding on the most rewarding flower colour on the second foraging trip, while bumblebees exposed at 2.6 and 10 ppb did not until their third and fifth trip, respectively. The delay in preference acquisition in exposed bumblebees may be due to reduced flower sampling and shorter foraging trips. These results show that bumblebees exposed to imidacloprid are slow to learn the reward value of flowers and this may explain previously observed foraging inefficiencies associated with pesticide exposure.

Keywords Bumblebee · Imidacloprid · Neonicotinoid · Learning · Memory · Foraging

Introduction

Insect pollinators provide a vital economic and ecological service, both increasing commercial crop yields (Klein et al. 2007; Ricketts et al. 2008) and sustaining wild plant populations (Ashman et al. 2004; Aguilar et al. 2006; Ollerton et al. 2011). Insect pollinators are thus important both to human wellbeing and to the functioning of natural ecosystems. A recent analysis valued global pollination services—most of which are carried out by insects—at approximately €153 billion per year (Gallai et al. 2009). It is, therefore, concerning that both wild and managed bee populations have been undergoing rapid and widespread declines (Goulson et al. 2008; Colla and Packer 2008; Ellis et al. 2010; Potts et al. 2010). Several anthropogenic environmental changes have been implicated in these declines, including pesticide use (Goulson 2013), land-use change (Kremen et al. 2007), the introduction of exotic species (Stout and Morales 2009), and pathogen spillover from commercially reared colonies (Otterstatter and Thomson 2008), all of which can interact to stress bee populations (Williams and Osborne 2009). Pesticides, for example, exacerbate the threat of natural pathogens to bee populations (Pettis et al. 2012; Prisco et al. 2013; Doublet et al. 2015).

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Neonicotinoids, currently the fastest growing class of insecticides (Jeschke et al. 2011), bind to nicotinic acetylcholine receptors in insects and lethally disrupt neural activity (Matsuda et al. 2001). Neonicotinoids are often applied as seed dressings, which then integrate into the tissue of growing plants and appear at levels ranging from < 1 to 10 parts per billion (ppb) in nectar and pollen (Sur and Stork 2003; Cresswell 2011; Blacquière et al. 2012; Stoner and Eitzer 2012; Godfray et al. 2015; Lundin et al. 2015). The median lethal dose (LD_{50}) value of imidacloprid, a common neonicotinoid, for the European honeybee, *Apis mellifera*, when ingested orally was found to be between 3.8 and 81 ng/bee (approximately 184–6000 ppb; Fairbrother et al. 2014). The LD_{50} value is similar for *Bombus impatiens*, a common North American bumblebee, when body size is accounted for (Scott-Dupree et al. 2009). Given that imidacloprid is typically found in crops at levels below 10 ppb (Cresswell 2011), exposure from crops is not having immediate lethal effects on bees (but see Girolami et al. 2012 and Samson-Robert et al. 2014 for evidence of other threatening forms of exposure). Evidence points instead to sublethal effects of neonicotinoids that impair the ability of bees to function on an individual or colony level (Goulson 2013). Field-realistic exposure to neonicotinoids negatively affects reproductive success (Tasei et al. 2000; Whitehorn et al. 2012; Laycock et al. 2012; Fauser-Misslin et al. 2014; Rundlöf et al. 2015; Woodcock et al. 2017), oocyte development, and colony initiation in bumblebee queens (Baron et al. 2017a, 2017b), pollen foraging (Gill et al. 2012; Gill and Raine 2014; Feltham et al. 2014; Stanley et al. 2016), learning and memory (Decourtye et al. 2004a, 2004b; Aliouane et al. 2009; Han et al. 2010; Williamson and Wright 2013; Stanley et al. 2015a), navigational skills (Henry et al. 2012; Matsumoto 2013), and thermoregulation (Tosi et al. 2016), and has been shown to induce neural apoptosis (Wu et al. 2015). This accumulation of sublethal effects could have a gradual but nonetheless damaging impact on bee populations by negatively affecting individual foragers, and subsequently, colony success. Further, sublethal effects of neonicotinoids on bee foraging behaviour can directly affect the quality of pollination bees provide to crops. Stanley and colleagues (2015b) found that exposed bumblebees visited fewer apple trees, collected less pollen from them, and that apples from trees pollinated by exposed bumblebees contained fewer seeds.

Although sublethal effects have been observed in both bumblebees and honeybees, there is good reason to believe that neonicotinoids pose a greater risk to bumblebees (Stoner 2016). Unlike honeybees, which shiver to produce heat and maintain colonies throughout winter, bumblebee colonies die off each season except for queens, which overwinter in diapause and reemerge to found new colonies when environmental conditions are suitable. Bumblebee population numbers, therefore, hinge on the number of queens produced at the end of each season. Neonicotinoid-induced stress has been shown to prevent the colony growth required to achieve high queen outputs (Whitehorn et al. 2012; Rundlöf et al. 2015). Further, it has been shown that exposed bumblebee queens develop smaller oocytes and are less likely to successfully initiate colonies (Baron et al. 2017a, 2017b). Baron and colleagues (2017b) show through Bayesian modelling that a neonicotinoid-induced reduction in colony initiation alone likely increases the probability of bumblebee population failure substantially.

How might neonicotinoids reduce bumblebee colony growth? A key finding is that exposed bumblebee workers return from foraging trips with less pollen—the key protein source for colony growth—than unexposed workers (Gill et al. 2012; Gill and Raine 2014; Feltham et al. 2014; Stanley et al. 2016). This suggests that neonicotinoid exposure negatively affects the foraging abilities of individual workers. A possible explanation for this is that neonicotinoid exposure disrupts learning and memory in workers, such that they cannot effectively retain information about rewarding food sources. Most studies addressing this possibility have used the proboscis extension reflex (PER), in which bees are removed from the colony, harnessed, conditioned to associate a stimulus with a sucrose reward, and later tested to see how well they remember the association. Studies using this paradigm have yielded mixed results regarding the effect of neonicotinoids on learning and memory in both bumblebees (Stanley et al. 2015a; Piiroinen et al. 2016) and honeybees (Williamson and Wright 2013; Tison et al. 2016; Piiroinen and Goulson 2016). There may be a difference, however, between success in the PER task and foraging success in the natural environment. Bees must retain a great deal of information when foraging in the wild, including the physical properties of flowers, their associated reward values, and the locations of flowers. To fully understand whether neonicotinoids disrupt learning and memory in bees, it may be necessary to use tasks that more closely resemble natural foraging conditions.

In the present study, we examined the effects of imidacloprid on learning and memory in the North American bumblebee *Bombus impatiens* in a context that resembles natural foraging. Exposed and unexposed bumblebee colonies were trained to forage in flight cages on artificial flowers containing 20% sucrose solution. Following this initial training, flowers of four different colours, each paired with a different sucrose concentration (10, 20, 30, and 40%) were presented. Bumblebees were tested for their ability to learn the associations between flower colour and reward and to forage preferentially on the highest sucrose concentration. It was hypothesized that if exposure to imidacloprid had a negative effect on learning and memory in worker

bumblebees then exposed bumblebees would show a weaker preference than unexposed bees for the flower colour associated with the highest sucrose concentration.

Materials and methods

Subjects and apparatus

Eight *Bombus impatiens* colonies of approximately 25–50 bees each were obtained from a commercial supplier (Biobest Canada Ltd, Leamington, ON). Colonies grew over the duration of the study to contain approximately 75–125 bumblebees. Behavioural measures were obtained from a total of 74 bees from these eight colonies; the mean number of bumblebees assessed per colony was 9.25, ranging from 1 to 16. Colonies were kept on a 12-h light: 12-h dark schedule (onset 0700 h) and housed in hive boxes connected to flight cages ($104 \times 64 \times 92$ cm) by 15 mm diameter polypropylene tubing. Control and imidacloprid-exposed bumblebees were held and tested in different but identical hive boxes and flight cages in separate rooms to prevent contamination of control bees with imidacloprid. Illumination was provided by 18–20 32 W Philips PLUS T8 tubes (F32T8/TL841 PLUS ALTO HV, Philips Lighting Holding B.V. ®). Each flight cage contained four patches of artificial flowers, each with four artificial flowers. Patches were rectangular sections of Styrofoam (20×32 cm) and artificial flowers were clear 1.7-ml microcentrifuge tubes (Axygen Inc., Union City, CA) with clear plastic corollas. Direct illumination upon artificial flowers measured using an illuminometer (YF-1065F Digital Illuminometer, Yu Fung Electronics Co.) was 910.96 ± 11.26 lux. The floor of the flight cage was covered in disposable surface protector (Whatman Benchkote®). Bumblebees foraged on clear artificial flowers *ad libitum* for 20% sucrose and the concentration of imidacloprid for that colony aside from during behavioural testing. Pollen was given to colonies directly in the hive box. Individual workers were given unique colour markings on the thorax using Posca paint markers (Mitsubishi Pencil Co.) so they could be identified during testing.

Pesticide preparation, exposure and detection

Imidacloprid in dry powder form (Imidacloprid PESTANAL Fluka 37894, Sigma-Aldrich) was dissolved in 20% sucrose solution to produce 2.6 and 10 ppb concentrations of imidacloprid. Sucrose concentration was verified using a refractometer. A stock solution of 50 ppm imidacloprid was prepared and diluted as necessary in 20% sucrose solution to make the 2.6 and 10 ppb imidacloprid solutions. All solutions were stored away from sunlight in amber tinted glass jugs to avoid ultraviolet (UV)-light exposure, which can cause imidacloprid breakdown (Soliman 2012).

Three colonies were chronically exposed to imidacloprid at 2.6 ppb, three were chronically exposed to imidacloprid at 10 ppb, and two were unexposed controls. The first colony at each exposure level fed for 2 weeks from a reservoir attached to the hive box on 20% sucrose solution containing the appropriate concentration of imidacloprid. These colonies were then connected to the flight cage in which bees foraged on artificial flowers containing the same sucrose and imidacloprid solution. The remaining colonies for each exposure level were connected to flight cages immediately upon arrival from the supplier and foraged for 20% sucrose with the appropriate imidacloprid solution in artificial flowers. These two different approaches were used to investigate whether there were differences in mortality between colonies initially exposed to imidacloprid while confined to the hive and colonies exposed in an active foraging setting. All colonies were given a minimum of 1 week to habituate to the flight cage before behavioural testing began. All sucrose solution consumed by bumblebees outside of testing contained the colony's respective imidacloprid treatment. This method of exposure was chosen to mirror the experience of bees foraging on imidacloprid-treated crops in bloom.

Following the completion of behavioural observations, liquid chromatography tandem-mass spectrometry (LC-MS/MS) was used to determine the concentration of imidacloprid and its metabolites in bumblebees from each colony using the analytical methods described by Gbylik-Sikorska and colleagues (2015). An amount of 2 ± 0.05 g of homogenous bumblebees from each colony were weighed in centrifuge tubes. The samples were extracted using 8 mL of acetonitrile and ethyl acetate mixture. After vortex mixing, samples were put in an ultrasonic bath to mix for 15 min, and then centrifuged. An aliquot of the supernatants was transferred to Sep-Pak Alumina N Plus Long cartridges without the preconditioned part. The supernatants were evaporated to dryness under a stream of nitrogen. The residues were dissolved in water and filtered into LC vials. Five µL of extracts were injected onto the LC-MS/MS instrument—Agilent 1200 HPLC system (Agilent Technologies, Germany) connected to the AB Sciex QTRAP® 6500 triple quadrupole mass spectrometer (AB Sciex, Canada). The chromatographic separation was performed on the Luna C18(2) column (Phenomenex, USA).

Behavioural task

Testing preparation

All bumblebees inside the flight cage were returned to the colony before each testing session. Circular coloured

corollas cut from Creatology™ foam sheets (Michaels Stores Inc.) were then placed over the clear corollas of artificial flowers. The coloured corollas were blue, green, orange, and yellow and they were placed such that each of the four artificial flower patches contained one flower of each colour. Any sucrose solution remaining in artificial flowers was removed, and flowers were refilled with 1.7 mL of sucrose solution such that blue, green, orange, and yellow flowers contained 10, 20, 30, and 40% imidacloprid-free sucrose solution, respectively. Sucrose concentrations were verified by refractometer.

Testing procedure

Marked bumblebees were allowed to enter the flight cage individually from the colony and were observed and timed while foraging on coloured flowers. Small metal blockades rounded at the bottom were placed through two slits in the polypropylene tube connecting the colony to the flight cage to regulate the flow of workers from the colony to the flight cage. Each time a bumblebee chose a flower to forage on, the colour of the flower, the time of the flower choice, and amount of time the bumblebee spent foraging on the flower was recorded. Flower choices were recorded in chronological order from the time the bumblebee entered the flight cage until the time it returned to the colony. A flower choice was defined as a flower visit during which the bumblebee extended its proboscis and consumed sucrose solution. The total duration of each foraging trip was recorded and artificial flowers were refilled to 1.7 mL between foraging trips. Each bumblebee was tested for 10 consecutive foraging trips. One to six bumblebees were tested during each observation session. Testing sessions took place weekly or biweekly for each colony.

Post-testing

Coloured corollas were removed from artificial flowers at the end of each testing session. Any remaining sucrose solution was removed from artificial flowers and they were refilled with 20% sucrose solution containing the colony's imidacloprid treatment. Bumblebees foraged *ad libitum* on artificial flowers with clear corollas until the next testing session.

Mortality assessment

The number of bumblebees found dead inside the flight cage was recorded daily. Mortality data were collected for six colonies over the first 3 weeks that bumblebees had access to the flight cage. Colonies assessed included two control colonies, two colonies exposed to imidacloprid at 2.6 ppb, and two colonies exposed to imidacloprid at 10 ppb. One colony from each exposure condition fed on sucrose solution from the reservoir attached to the colony for 2 weeks prior to having flight cage access, whereas the other colony from each exposure condition was given flight cage access immediately upon arrival from the supplier.

Table 1 Number of bumblebees at each imidacloprid exposure level observed for each behavioural measure

Behavioural measure	Number of bumblebees observed		
	0 ppb	2.6 ppb	10 ppb
Time on flowers types	22 of 28	18 of 18	28 of 28
Choice of flower types	28 of 28	14 of 18	28 of 28
Foraging trip duration	28 of 28	18 of 18	28 of 28
Number of choices	28 of 28	18 of 18	28 of 28
Time foraging on flowers	22 of 28	18 of 18	28 of 28
Latency	20 of 28	14 of 18	28 of 28

Data analysis

Behavioural data were collected from a total of 74 worker bumblebees, although data were not collected from all bumblebees on all behavioural measures. See Table 1 for the number of bumblebees from each imidacloprid exposure group observed for each measure of behaviour. SPSS (IBM Corp. Released 2016. IBM SPSS Statistics for Macintosh, Version 24.0. Armonk, NY) was used for all analyses, and analyses were conducted using an alpha level of 0.05. Post hoc tests were conducted a posteriori using the Bonferroni correction. Behavioural data for all subjects are available as supplementary material (Online Resource 1).

The time bumblebees spent foraging on flowers of each colour, as well as the relative frequency with which bumblebees chose flowers of each colour were each analyzed using a repeated-measures analysis of variance (ANOVA) with the between-subjects factor of imidacloprid concentration (0, 2.6, and 10 ppb) and the within-subjects factors of artificial flower colour (blue, green, orange, and yellow) and trial (1–10). Relative choice frequency for each flower type was calculated by dividing the number of times bumblebees sampled each type of flower by the total number of times bumblebees sampled flowers during each trial. No data were excluded from the analysis of time spent on different flower types, however, for data to be included in the analysis of relative flower type choice frequency, bees had to make at least one flower choice on all trials. The statistics reported from each of these analyses have been Greenhouse–Geisser corrected, as the assumption of sphericity was not met.

The total duration of foraging trips, number of flower choices, latency to choose an initial flower, and total time spent on flowers by bumblebees were each analyzed using a

repeated-measures ANOVA with the between-subjects factor of imidacloprid concentration (0, 2.6, and 10 ppb) and the within-subjects factor of trial (1–10). No data were excluded for total duration of foraging trips, number of flower choices, or time spent on flowers, however, for data to be included in the analysis of latency to choose an initial flower, bees had to make at least one flower choice on all trials. The statistics reported from these analyses have been Greenhouse–Geisser corrected, as the assumption of sphericity was not met.

Two chi-square analyses were used to test whether the number of bumblebees found dead in the flight cage differed among the six colonies for which deaths were recorded. The first chi-square analysis compared the number of bumblebees found dead the day after colonies gained access to the flight cage. The second chi-square analysis compared the number of bumblebees found dead on the 15th day of the imidacloprid exposure period; this was the 15th day of flight cage access for unconfined colonies given immediate access to the flight cage, and the second day of flight cage access for colonies that were confined to the hive box for 2 weeks before flight cage access. Mortality data for the six colonies are available as supplementary material (Online Resource 1).

Results

Verifying imidacloprid presence in bees

LC-MS/MS revealed the presence of imidacloprid-urea, a metabolite of imidacloprid, in bumblebee samples from all three colonies exposed to imidacloprid at 10 ppb, one of the three samples from the three colonies exposed at 2.6 ppb, and neither of the two samples from the two unexposed colonies (Table 2).

Table 2 Imidacloprid-urea detected in a bumblebee sample from each of eight colonies

	Confined			Unconfined				
Imidacloprid exposure (ppb)	0	2.6	10	0	2.6	2.6	10	10
Imidacloprid-urea (ng/g)	0	0	1.8	0	0.12	0	5.8	4

Values in the imidacloprid exposure row indicate exposure concentrations for each of the eight colonies. Values in the imidacloprid-urea row show the concentration of imidacloprid-urea in a single 2 ± 0.05 g homogenous sample of bumblebees from each colony. Bumblebees in Confined colonies fed for 2 weeks from a reservoir of sucrose containing imidacloprid attached to the colony before entering the flight cage. Bumblebees in Unconfined colonies entered the flight cage immediately. See text for details

Behavioural task

Time spent foraging on differently coloured flowers

There was a significant interaction among imidacloprid concentration, trial, and colour, indicating that the groups differed in how their preferences for which flowers to consume sucrose solution from developed over the 10 trials ($F(18.20, 591.53) = 1.619$, $p = 0.05$). Unexposed bumblebees developed a slight preference for feeding on yellow flowers (containing 40% sucrose solution) on the second trial (Fig. 1a), bumblebees exposed to imidacloprid at 2.6 developed a preference for yellow flowers on the third trial (Fig. 1b)—although this preference was stronger than that developed by unexposed bumblebees—and bumblebees exposed to imidacloprid at 10 ppb did not develop a preference for yellow flowers until the fifth trial (Fig. 1c). Once the preference for feeding on yellow flowers occurred, it was maintained by bumblebees from all exposure conditions. There were significant main effects of colour ($F(1.25, 81.45) = 88.874$, $p < 0.001$) and trial ($F(4.53, 294.37) = 3.535$, $p < 0.001$). There was also a significant interaction between colour and trial ($F(9.1, 591.53) = 10.076$, $p < 0.001$) showing that flower colour preferences changed across trials. No significant main effect was found for imidacloprid concentration ($F(2, 65) = 0.685$, $p = 0.508$) and there were no significant interactions between trial and imidacloprid concentration ($F(9.06, 591.53) = 1.692$, $p = 0.090$) or between colour and imidacloprid concentration ($F(2.506, 591.53) = 6.097$, $p = 0.081$). An additional figure showing the time bumblebees spent foraging on different coloured flowers by colony at each exposure level is available as supplementary material (Online Resource 2). Although there are differences between colonies at each exposure level, no colonies exposed to imidacloprid at 10 ppb showed a preference for yellow flowers prior to trial four, whereas all other colonies displayed a fairly strong preference for yellow flowers by trial three, with the exception of one control colony.

Relative choice frequency of differently coloured flowers

There was a significant main effect of imidacloprid concentration, indicating that imidacloprid exposure influenced the relative frequency with which bumblebees chose differently coloured flowers while foraging ($F(2, 67) = 4.262$, $p = 0.018$; Fig. 2a–c). A post hoc test using the Bonferroni correction showed that bumblebees exposed to imidacloprid at 2.6 and 10 ppb differed significantly at $p < 0.05$, whereas unexposed bumblebees did not differ significantly from bumblebees exposed at 2.6 or 10 ppb; bumblebees from colonies exposed at 2.6 ppb displayed a strong preference for yellow flowers by trial three, and those exposed at 10 ppb displayed a fairly strong preference for orange flowers until trial eight. Although there was a significant

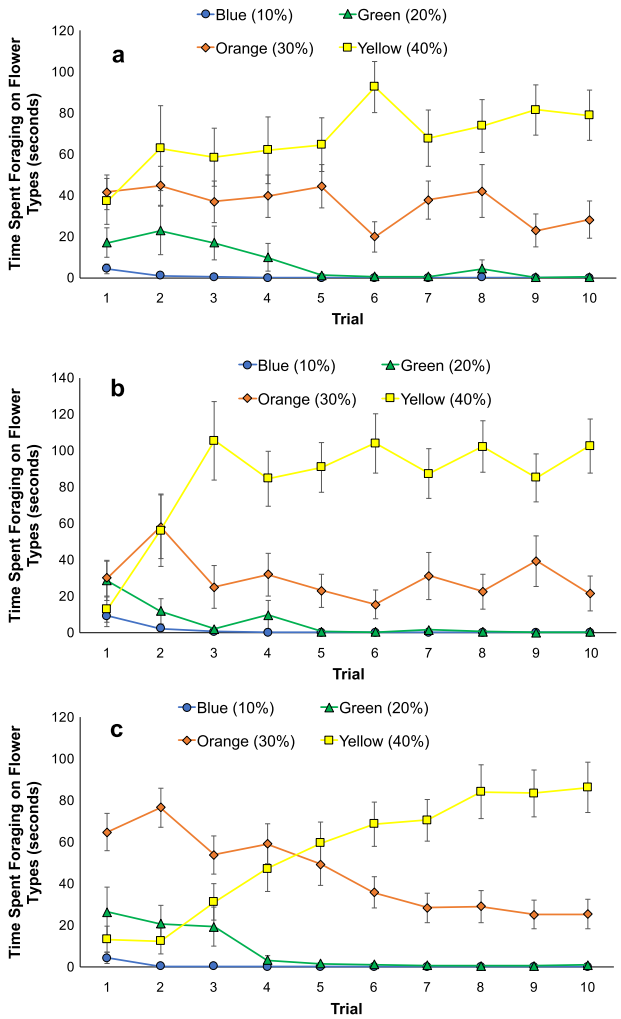

Fig. 1 Time spent foraging on four different flower types: green (10% sucrose), blue (20% sucrose), orange (30% sucrose), yellow (40% sucrose). **a** Unexposed bumblebees developed a slight preference for feeding on yellow flowers on the second trial, **b** bumblebees exposed to imidacloprid at 2.6 ppb developed a preference for yellow flowers on the third trial, and **c** bumblebees exposed to imidacloprid at 10 ppb did not develop a preference for yellow flowers until the fifth trial. Error bars represent one standard error of the mean

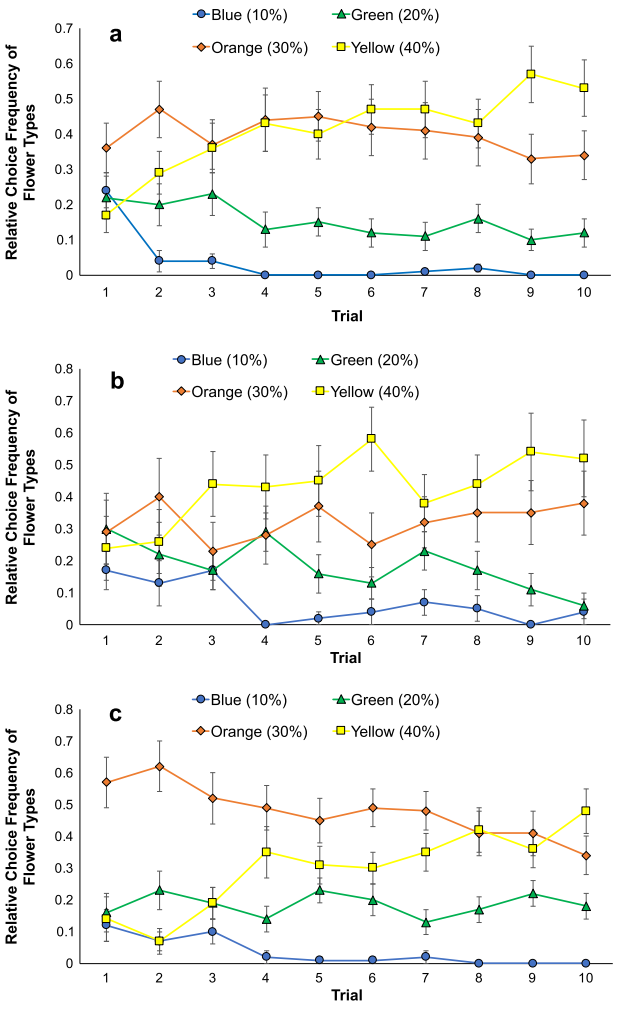

Fig. 2 Relative choice frequency for four different flower types: green (10% sucrose), blue (20% sucrose), orange (30% sucrose), yellow (40% sucrose). **a** Unexposed bumblebees chose orange and yellow flowers at a similar rate, whereas **b** bumblebees exposed to imidacloprid at 2.6 ppb displayed a preference for yellow by trial three and **c** bumblebees exposed at 10 ppb displayed a preference for less rewarding orange flowers until trial eight. Error bars represent one standard error of the mean

main effect of colour ($F(1.54,\ 103.39) = 49.765$, $p < 0.001$), there was no significant main effect of trial ($F(6.28,\ 420.98) = 1.538$, $p = 0.131$). However, there was a significant interaction between colour and trial, indicating that preferences for differently coloured flowers shifted across trials ($F(13.18,\ 882.81) = 4.218$, $p < 0.001$); overall, bumblebees visited fewer blue and green flowers following early trials and instead visited more rewarding orange and yellow flowers. There were no significant interactions between trial and imidacloprid concentration ($F(12.57,\ 882.81) = 0.418$, $p = 0.961$), colour and imidacloprid concentration ($F(3.09,\ 882.81) = 2.394$, $p = 0.071$), or trial, colour and imidacloprid concentration ($F(26.35,\ 882.81) = 0.863$, $p = 0.665$). An additional figure showing the relative choice frequency of bumblebees for different coloured flowers by colony at each exposure level is available as supplementary material (Online Resource 3). Although there are differences between colonies in relative flower choice frequency, at least one colony exposed to imidacloprid at 0 or 2.6 ppb displayed a preference for visiting yellow flowers by trial three, whereas no colonies exposed at 10 ppb showed such a preference before trial four.

Duration of foraging trips

There was a significant interaction between imidacloprid concentration and trial in the mean duration of foraging trips ($F(5.24,\ 185.92) = 4.455$, $p = 0.001$; Fig. 3). Post hoc tests

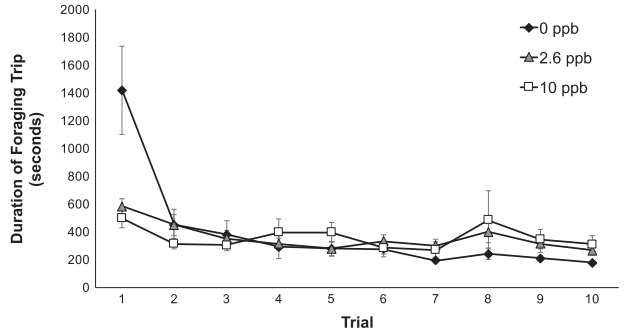

Fig. 3 Duration of foraging trips. Bumblebees exposed to imidacloprid at either 2.6 or 10 ppb spent less time foraging on the first trial than unexposed bumblebees. Error bars represent one standard error of the mean

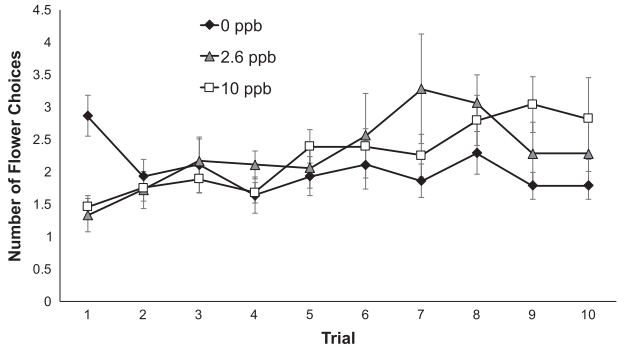

Fig. 4 Number of flower choices. Bumblebees exposed to imidacloprid at either 2.6 or 10 ppb visited fewer flowers on the first trial than unexposed bumblebees. Error bars represent one standard error of the mean

using the Bonferroni correction showed that unexposed bumblebees spent significantly more time foraging during trial one than bees exposed to imidacloprid at each 2.6 and 10 ppb at $p < 0.05$. Unexposed bumblebees made slightly but nonsignificantly shorter foraging trips relative to exposed bumblebees following trial six. There was no significant main effect of imidacloprid concentration ($F(2, 71) = 0.127$, $p = 0.881$), but there was a main effect of trial ($F(2.62, 185.92) = 10.281$, $p < 0.001$) showing that in general, foraging trip duration decreased across trials regardless of imidacloprid concentration.

Number of flower choices

There was a significant interaction between imidacloprid concentration and trial in the number of flower choices made ($F(11.17, 396.36) = 2.219$, $p = 0.012$; Fig. 4). Post hoc tests using the Bonferroni correction showed that unexposed bumblebees foraged on significantly more flowers during trial one than bumblebees exposed to imidacloprid at each 2.6 and 10 ppb at $p < 0.05$. Unexposed bumblebees foraged on slightly but nonsignificantly fewer flowers following trial six. There was no significant main effect of imidacloprid concentration ($F(2, 71) = 0.661$, $p = 0.546$), but there was a significant main effect of trial ($F(5.58, 396.36) = 2.490$, $p = 0.026$) indicating that in general the number of flower choices tended to increase across trials.

Time foraging on flowers

There was no significant interaction between imidacloprid concentration and trial in the total time spent foraging on flowers ($F(9.13, 296.62) = 1.751$, $p = 0.076$; Fig. 5). There was a significant main effect of trial ($F(4.56, 296.62) = 3.691$, $p = 0.004$) showing that the amount of time bumblebees spent consuming sucrose changed across trials overall; most of this effect appears due to an increase

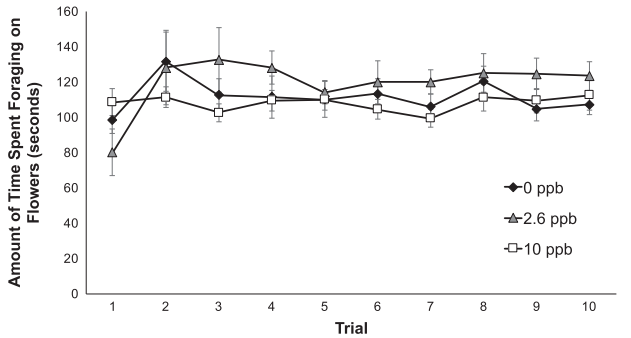

Fig. 5 Total time spent foraging on flowers. The total amount of time bumblebees spent foraging on flowers increased across trials but all bumblebees spent similar amounts of time foraging. Error bars represent one standard error of the mean

between the first trial and subsequent trials in time spent foraging on flowers. There was no significant main effect of imidacloprid concentration ($F(2, 65) = 0.748$, $p = 0.477$).

Latency to choose an initial flower

There was no significant interaction between imidacloprid concentration and trial in the latency to choose an initial flower ($F(2.51, 74.11) = 1.939$, $p = 0.140$; Fig. 6). Unexposed bumblebees showed a longer but nonsignificant latency to choose an initial flower on the first trial. There was a significant main effect of trial ($F(1.26, 74.11) = 9.475$, $p = 0.001$), showing that latency to choose the first flower decreased across trials. There was no significant main effect of imidacloprid concentration ($F(2, 59) = 0.531$, $p = 0.591$).

Mortality

The number of bumblebees from each colony found dead in the flight cage over the first 3 weeks of flight cage access is shown in Fig. 7. We compared the number of bumblebees

found dead the day after they were given access to the flight cage, shown as day 1 for unconfined bumblebees and day 15 for confined bumblebees in Fig. 7. Significantly more bumblebees from colonies confined for 2 weeks and exposed to imidacloprid at 2.6 or 10 ppb were found dead the day after gaining flight cage access ($X^2(5) = 173.94$, $p < 0.001$). In addition, we conducted a second distinct comparison that focused not on mortality during the first day of flight cage access for all colonies, but instead focused on day 15 of imidacloprid exposure for all colonies. Significantly more bumblebees from colonies confined for 2 weeks and exposed to imidacloprid at 2.6 or 10 ppb were found dead on the 15th day of imidacloprid exposure ($X^2(5) = 173.94$, $p < 0.001$). Mortality was minimal for all six colonies on other days.

Discussion

We found that chronic exposure to imidacloprid affected in a dose-dependent manner the rate at which bumblebees learned associations between flower colour and sucrose concentration and foraged preferentially on flowers with the greatest sucrose concentration. The major effect of imidacloprid exposure was a delay in the development of a preference to forage on flowers preferred by unexposed bumblebees. Although bumblebees exposed at 2.6 ppb displayed a preference for highly rewarding yellow flowers (containing 40% sucrose) on trial three—one trial later than unexposed bees—bumblebees exposed to imidacloprid at 10 ppb did not preferentially feed on yellow flowers until trial five. Exposed bumblebees differed from unexposed bumblebees in the duration of their initial foraging trips, and the number of flowers they sampled during initial foraging trips. Previous studies of the effects of neonicotinoids on learning and memory in bees have shown reduced responses to rewarded olfactory cues (Decourtye et al. 2004b; Williamson and Wright 2013; Stanley et al. 2015a, b), and spatial memory impairment (Samuelson et al. 2016). The current study provides evidence for learning deficits in which cues to nectar concentration are visual. Furthermore, bees in our study were tested in a relatively natural foraging context in which they could fly freely between the colony and a flight cage containing artificial flowers, collecting nectar, and returning to the colony between trials. This allowed us to avoid the possible stress of removal from the colony and harnessing as is required in PER studies. We also detected imidacloprid-urea, a metabolite of imidacloprid, following behavioural testing in bumblebees from all colonies exposed to imidacloprid at 10 ppb, and at a relatively small amount in one of the three colonies exposed at 2.6 ppb. This provides evidence that bumblebees consuming imidacloprid did retain metabolites in a dose-dependent fashion, although it is interesting that imidacloprid-urea was only detected in one of the colonies exposed at 2.6 ppb. A likely explanation for this is that bumblebee samples were collected following substantial metabolite decay. Research

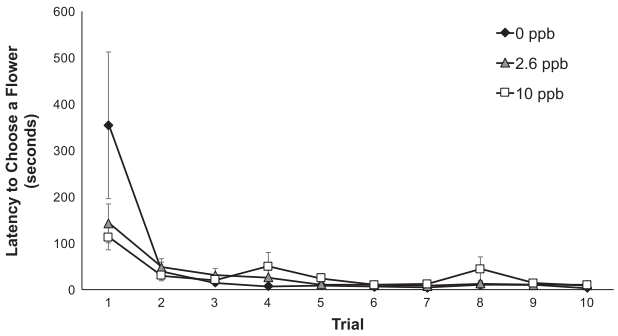

Fig. 6 Latency to first flower choice. All bumblebees showed a similar latency to their first flower choice. The difference between unexposed and imidacloprid-exposed bumblebees on the first trial is not significant. Error bars represent one standard error of the mean

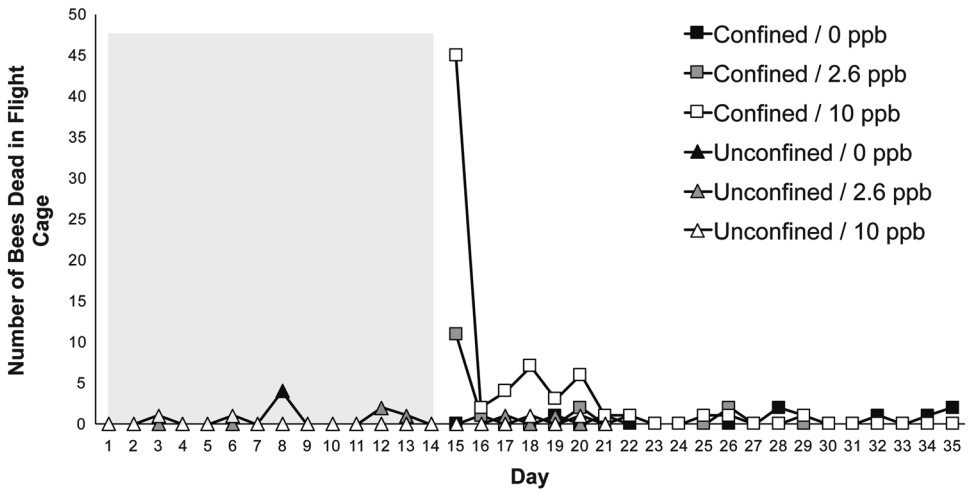

Fig. 7 Number of bumblebees found dead in the flight cage. Confined bumblebees were exposed to imidacloprid in the colony from days 1 to 14 (shaded) and did not have flight cage access. Unconfined bumblebees fed on sucrose containing imidacloprid in artificial flowers in the flight cage from days 1 to 14. Mortality was significantly elevated for confined imidacloprid-exposed bumblebees following their first day of flight cage access

with honeybees has shown that imidacloprid is rapidly metabolized, and that approximately 90% of metabolites decay within 72 h. Neonicotinoid molecules bound to nicotinic acetylcholine receptors likely decay at a slower rate (Suchail et al. 2004; Rondeau et al. 2014). Although it is possible that neonicotinoid metabolites may still have been present in bumblebees from other colonies exposed at 2.6 ppb, they were below our threshold of detection.

Bumblebees chronically exposed to imidacloprid at 2.6 and 10 ppb did not display a preference for yellow flowers until trials three and five, respectively, whereas unexposed bumblebees displayed a slight preference for yellow flowers by trial two. Despite this delay in exposed bees' acquisition of a preference for yellow flowers, bees from all exposure groups eventually acquired and maintained the preference for yellow flowers. This suggests that the deficit observed in imidacloprid-exposed bumblebees was specific to acquisition and not retention of memory for the association between flower colour and sucrose concentration. It has been shown that learning deficits have important implications for colony success. Raine and Chittka (2008) found a direct correlation between bumblebees' ability to learn flower colour-sucrose reward associations and how much nectar the colonies collected in field conditions. Imidacloprid-induced learning deficits may, therefore, affect the amount of resources colonies are able to collect and devote to colony growth. Furthermore, Müller and Schmid-Hempel (1992) found that typically only the largest bumblebee colonies produce new queens at the end of the season. Given that bumblebee populations depend on new queens produced at the end of each season, learning deficits induced by imidacloprid exposure could reduce the collection of resources, reduce colony growth, and in this way, play an important role in population declines.

Although it is clear that imidacloprid exposure slowed the rate at which exposed bumblebees displayed a preference for rewarding yellow flowers when using a measure of time spent feeding on flowers, the delay is less clear when using a measure of relative choice frequency. We found that bumblebees exposed to imidacloprid at 2.6 ppb displayed a preference for yellow flowers (containing 40% sucrose) by trial three, unexposed bees spent most trials sampling equally between orange and yellow flowers, and bumblebees exposed at 10 ppb preferred less rewarding orange flowers (containing 30% sucrose) until trial eight. Preferences using this measure were likely less clear because bumblebees continued to sample flowers across trials without settling to feed on them. It is important to note, however, that perceptual confusion may explain why preferences on this measure were less consistent. Although bumblebees quickly rejected less rewarding blue and green flowers, they may have been slower to reject orange flowers due to perceived similarity between orange and yellow. Bumblebees are visually less sensitive to colours in the red zone relative to the UV, blue, and green zones (Skorupski and Chittka 2010), and it has been shown that bumblebees require a number of differential conditioning trials in order to successfully discriminate between perceptually similar colours (Dyer and Chittka 2004). It is possible that imidacloprid-induced deficits in perceptual discrimination could explain why bumblebees exposed at 10 ppb did not display a preference for yellow flowers until trial 10 using a measure of relative choice frequency.

We found that bumblebees chronically exposed to imidacloprid at both 2.6 and 10 ppb differed from unexposed bees in the duration of foraging trips and the number of flowers sampled in early trials. Bumblebees exposed to imidacloprid at both levels spent less than half the time in the flight cage as unexposed bumblebees during trial one and sampled only half as many flowers. This lack of exploratory behaviour displayed by exposed bumblebees relative to unexposed bumblebees could explain why exposed bumblebees acquired a preference for the highly rewarding yellow flowers at a slower rate. Reduced sampling could prevent bumblebees from learning about the full range of flowers and their associated reward levels, and result in exposed foragers having less information on which to base their floral preferences when foraging. Evans and Raine (2014) demonstrated the importance of flower sampling, showing that bumblebees continually sampling different flowers in a stable array of artificial flowers were faster to discover a rewarding novel flower type when it was introduced into the array. The costs of sampling were negligible in our simplified foraging environment and, given the 10–40% range in sucrose concentrations available, far outweighed by the benefits of learning the predictive association between flower colour and sucrose concentration. We found no effect of imidacloprid exposure on the total amount of time bumblebees spent consuming sucrose solution across trials, suggesting that bumblebees from all groups were equally motivated to forage, and that reduced exploratory behaviour did not result from reduced motivation (but see Thompson et al. 2015 for antifeedant effects of neonicotinoids).

Imidacloprid-induced motor deficits could explain the reduced exploratory behaviour we observed in exposed bumblebees. Imidacloprid disrupts neural activity in insects by blocking nicotinic acetylcholine receptors in the brain (Matsuda et al. 2001), and while affected receptors are often identified in brain areas involved in learning and memory such as the mushroom bodies and the antennal lobe (Barbara et al. 2008; Palmer et al. 2013; Moffat et al. 2015), motor deficits have also been observed following sublethal exposure. Williamson and colleagues (2014) found that honeybees were much slower to right themselves after falling, suggesting coordination deficits that could have a

broad impact on more complex aspects of foraging such as navigation (Henry et al. 2012; Matsumoto 2013; but see Stanley et al. 2016) and pollen acquisition (Gill et al. 2012; Gill and Raine 2014; Feltham et al. 2014; Stanley et al. 2016). Exploratory behaviour may be motorically more effortful for imidacloprid-exposed bumblebees, causing exposed bumblebees in our study to stop and collect sucrose solution from the first flower they landed on rather than sample multiple flower types during early trials, though they did visit more flowers during later trials. It is also worth noting that exposed bumblebees appeared quicker to choose a flower in initial trials, although this effect was not significant.

We found a striking difference in mortality between confined imidacloprid-exposed colonies and other colonies following the first day that confined imidacloprid-exposed colonies were given access to the flight cage. Confined colonies consumed either 2.6 or 10 ppb 20% sucrose solution from a reservoir attached to the hive box and were not given access to the flight cage for 14 days. Unconfined colonies fed from the same 2.6 or 10 ppb 20% sucrose solution in artificial flowers in the flight cage. We observed 11 and 45 dead bumblebees from confined colonies exposed to imidacloprid at 2.6 and 10 ppb, respectively. There was very little mortality in unconfined imidacloprid-exposed colonies and control colonies (Fig. 7). Although this result is very preliminary it suggests there may be an interaction between flight activity and imidacloprid exposure in which a period of inactivity followed by free flight raises mortality in imidacloprid-exposed bumblebees. It was not obvious from daily observations of each colony that bees were dying inside the colonies. It is also possible, however, that morbidity occurred in exposed bumblebees while confined in the colony box, and that once flight cage access was available these bees left the colony to die or were removed by other colony members.

Sucrose solution was easy to obtain from artificial flowers in this study. Real flowers can be more complex and bumblebees often have to learn complex motor patterns to successfully collect pollen from flowers (Laverty 1994; Raine and Chittka 2007). Pollen is the key protein source for colony growth, and it has been well-established that neonicotinoid-exposed bumblebees collect less of it (Gill et al. 2012; Gill and Raine 2014; Feltham et al. 2014; Stanley et al. 2016). To determine how neonicotinoids affect bumblebee foraging success, it would be valuable to examine more complex motor tasks that closely resemble nectar and pollen collection in the wild. Recent studies have found that exposed bumblebees take more trials to learn how to efficiently handle flowers (Stanley and Raine 2016), and show decreased sonication, or buzz pollination (Switzer and Combes 2016). In addition, bumblebees have been shown to learn different floral colour and reward associations for nectar and pollen simultaneously (Muth et al. 2015). It would be valuable to determine how the acquisition and retention of multiple flower colour and reward associations over the weeks of a forager's lifespan are affected by neonicotinoid exposure.

There are several limitations in the current study worth noting. First, colour–reward combinations were identical for all bumblebees tested. Varying colour–reward combinations across subjects would control for initial colour preferences and perceptual similarity of colours. This would make it possible to determine whether flower preferences develop differently depending on colour, sucrose concentration, and the combination of the two. Second, although we believe there are advantages in terms of externally validity to assessing neonicotinoid effects in bumblebee workers from queenright colonies, the use of this method does make it more difficult to control for colony variation. Although variation between colonies within each exposure group occurred, colonies did show similar trends. Notably, all colonies exposed to imidacloprid at 10 ppb developed a preference for the most rewarding flower type at a relatively slow rate according to measures of time on flowers and choice frequency.

In summary, we found that imidacloprid exposure affected the rate at which bumblebees learned to feed preferentially on highly rewarding flowers. Although bumblebees exposed at 2.6 ppb showed a preference for the most rewarding flower type on trial three—one trial after unexposed bumblebees—they maintained a stronger preference than unexposed bumblebees. However, bumblebees exposed at 10 ppb did not display a preference for the most rewarding flower type until the fifth trial. Interestingly, although both unexposed bumblebees and those exposed to imidacloprid at 2.6 ppb showed an early preference for feeding on the most rewarding flower type, many continued to sample less rewarding flowers, perhaps as a result of perceptual confusion, before moving on to more rewarding flowers. Exposed bumblebees spent significantly less time foraging during their first trial and sampled far fewer flowers than unexposed bees, which likely contributed to their delayed preference for highly rewarding flowers. In the wild, bumblebees encounter a foraging environment more challenging than learning about four distinctively coloured flower types in a spatially small area. Bumblebees often travel great distances to forage on flowers that differ visually, olfactorily and morphologically and vary in nectar production on a daily and seasonal basis (Zimmerman and Pyke 1986; Real and Rathcke 1988). Our results show that imidacloprid exposure can slow the rate of learning about this foraging environment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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

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