

## Research



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### Author for correspondence:

Felicity Muth  
e-mail: [felicity.muth@austin.utexas.edu](mailto:felicity.muth@austin.utexas.edu)

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## Animal behaviour

# Modality-specific impairment of learning by a neonicotinoid pesticide

Felicity Muth<sup>1</sup>, Jacob S. Francis<sup>2</sup> and Anne S. Leonard<sup>2</sup>

<sup>1</sup>Department of Integrative Biology, University of Texas at Austin, 2415 Speedway, Austin, TX 78712, USA

<sup>2</sup>Department of Biology, University of Nevada, Reno, NV 89557, USA

FM, 0000-0003-0904-0589

Neonicotinoid pesticides can impair bees' ability to learn and remember information about flowers, critical for effective foraging. Although these effects on cognition may contribute to broader effects on health and performance, to date they have largely been assayed in simplified protocols that consider learning in a single sensory modality, usually olfaction. Given that real flowers display a variety of potentially useful signals, we assessed the effects of acute neonicotinoid exposure on multimodal learning in free-flying bumblebees. We found that neonicotinoid consumption differentially impacted learning of floral stimuli, impairing scent, but not colour, learning. These findings raise questions about the mechanisms by which pesticides might differentially impair sensory systems, with implications for how neonicotinoids affect multiple aspects of bee ecology.

## 1. Introduction

Honeybees (*Apis*) and bumblebees (*Bombus*) are ecologically and agriculturally critical pollinators that also serve as important model systems for the study of cognition [1]. As generalist foragers, individuals visit a variety of flower types and rapidly learn associations between floral cues and rewards such as nectar [2–5] and pollen [6–8]. This ability to learn associations is an important aspect of foraging performance, potentially linked to fitness ([9], but see [10]). Stressors such as pathogens, poor nutrition and pesticide exposure can have negative effects on bee learning [11–15], and thus have been highlighted as being potentially detrimental to foraging efficiency and colony growth [16].

Of these stressors, neonicotinoid pesticides have been an area of recent focus for their impacts on bee cognition. These pesticides are widely used (e.g. in the USA and China [17,18]), where crops can be seed- or spray-treated. As systemic pesticides, neonicotinoids are expressed in plants' nectar and pollen, where foraging bees can ingest them [19]. While many studies have found detrimental effects of these pesticides on learning (e.g. [16,20], reviewed in [14]), others have found impairment to other components of foraging behaviour such as motivation, but not learning *per se* [21,22].

Nearly all of the previous work on how neonicotinoids impact cognition addresses learning in a single sensory modality (usually olfaction: see Fig. 1 in [21]). However, these single-modality tests are not representative of natural foraging scenarios, since bees use multiple sensory modalities to locate and learn about flowers [23]. For example, components of multimodal floral displays can have interactive effects on bee learning [24], and floral colour can compensate in scenarios where scent is less useful [25]. The single-modality focus of pesticide research may explain discrepancies in recent reports of neonicotinoids' impact on bee foraging [21]. Specifically, where studies have found neonicotinoid-induced learning impairment, they have generally addressed olfactory learning (e.g. [16,20,26–28]), while studies on visual learning have found either no effects [21,22], or effects consistent with other explanations [29].

However, these discrepancies could also be explained by differences in training protocol: olfactory learning is usually studied using harnessed bees in the proboscis extension response (PER) protocol [30,31], whereas visual learning is more commonly studied using free-flying bees. Thus, addressing whether impairment from neonicotinoids might be modality-specific requires learning be measured under the same conditions.

To explore whether bees' olfactory and visual learning might be differentially impaired by a neonicotinoid, we assessed the effects of imidacloprid on multimodal learning in free-flying bumblebees, *Bombus impatiens*. We trained individuals to discriminate between flowers that differed in scent and colour cues before measuring performance in a test phase where flowers offered all possible combinations of the trained scents and colours, including flowers where these cues were put into conflict (figure 1). We expected that if scent and colour learning were differentially impaired by imidacloprid exposure, exposed bees would make more of one type of error (selecting the floral option with the incorrect scent or colour) compared with the control bees (figure 2b). Alternatively, if scent and colour learning were impaired equally, we expected that errors of each type would be equally common in control and imidacloprid-treated bees; this could also indicate an impairment to the ability to combine scent and colour stimuli (i.e. multisensory integration [23]).

## 2. Methods

### (a) Subjects

We tested 60 *Bombus impatiens* individuals from three colonies (Koppert Biological Systems, USA; electronic supplementary material, table S1). Colonies were sequentially connected to a foraging arena ( $L \times W \times H$ : 122 × 59 × 59 cm), via a gated passage-way and trained to forage (during a 'shaping' phase) on a horizontal array of artificial flowers. We maintained colonies on 30% (w/w) sucrose pipetted directly into honeypots after each testing day, and approximately 500 mg honeybee-collected pollen (Koppert Biological Systems, USA) added to each colony every 2–3 days. We paint-marked foragers; details on shaping, arena, and artificial flowers are in the electronic supplementary material.

### (b) Pesticide treatment

Imidacloprid solutions were only ever fed to individual bees before trial 1. We caught foragers as they left the colony by allowing them to walk into a dark holding container (electronic supplementary material, figure S1a) and fed them either 0.45 ng (22.5 ppb) imidacloprid-containing or control solutions, in 20  $\mu$ l of 30% (w/w) sucrose (details in electronic supplementary material). This dose is in line with field-exposed plants' nectar, which can range from 1 to 50 ppb, with maximum documented concentrations of 912 ppb [32,33]; see also Table S2 in Siviter *et al.* [14] for average and maximum estimates of neonicotinoid concentrations in nectar ingested for a given bumblebee foraging bout. Our dose is also in line with previous experimental work on bee behaviour [14]. All foragers consumed the solution, and were held in the container for 1 h before trial 1 to maximize pesticide absorption, in line with previous behavioural studies [20,21,34].

### (c) Training and testing

We trained individual bees over two training trials (inter-trial interval approximately 5 min), followed by a test 10 min later.

For each trial, we gave a bee access to a 48-flower array containing two artificial floral types: purple scented with linalool and blue scented with geraniol (electronic supplementary material, figure S1b,d,e). Each flower type contained either 4  $\mu$ l of 50% (w/w) sucrose (rewarding flowers) or 4  $\mu$ l of water (unrewarding flowers). Stimuli–reward pairings were counterbalanced across colonies and pesticide treatment (electronic supplementary material, table S1). We cleaned flower tops with 70% ethanol between trials and changed flower locations between each training trial and bee.

At the start of trial 1, we transferred the bee to a single white flower, identical to the ones used during the shaping phase, containing 4  $\mu$ l of 50% (w/w) sucrose solution, and stimulated the bee's antennae with 50% (w/w) sucrose to induce proboscis extension. All bees consumed this sucrose, which was offered in order to motivate bees to initiate foraging after having been held in the dark. Bees then free-foraged on the array; we refilled visited flowers after bees visited three to six subsequent flowers. We ended trials when a bee returned to the colony via the connecting tube or left the array for more than 2 min (we then manually returned it to the colony). Trial 2 was run identically to trial 1. If bees did not return for trial 2, we transferred them to the arena and in most cases the bee then resumed foraging (electronic supplementary material, table S1). For the test trial, we presented bees with a 48-flower array with four flower types, all offering 4  $\mu$ l of water (electronic supplementary material, figure S1c), representing the four possible combinations of the two colours and scents (figure 1), and allowed bees to make 20 visits.

### (d) Behaviour coding and data analyses

We filmed training and test trials from above using an HD Sony camcorder (30 fps). From the videos we recorded the type of flower visits bees made (Solomon Coder; <https://solomoncoder.com>): 'rewarding' (consumes sucrose), 'unrewarding' (probes water), 'empty' (probes previously emptied well) or 'does not drink' (lands on the flower but does not probe the well). We analysed bees' learning and test performance, as well as their motivation (tendency to forage in trial 1 and to return to forage in trial 2) and foraging performance (see electronic supplementary material). All analyses were carried out in R v.3.4.3. [35].

## 3. Results

### (a) Training trial behaviour

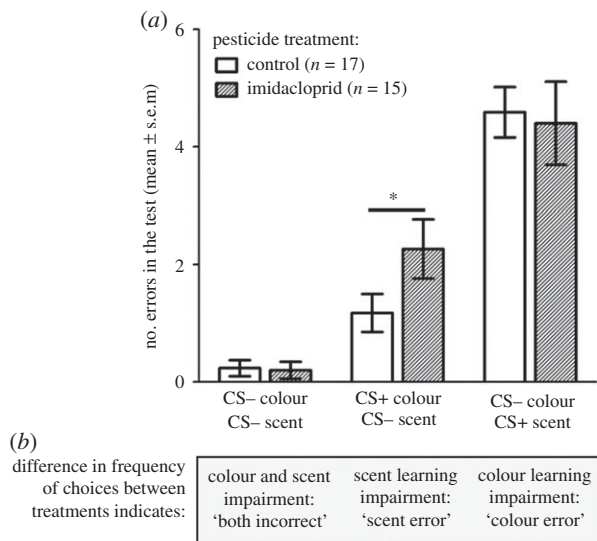
Regardless of pesticide treatment, bees learned across the two trials, performing better in trial 2 than trial 1 ( $z = 9.444$ ,  $p < 0.0001$ ; electronic supplementary material, figure S2a). Imidacloprid-treated bees performed worse than control bees ( $z = 2.504$ ,  $p = 0.0123$ ), with this effect being driven by the treatment where bees were trained that blue/geraniol flowers were rewarding (pesticide treatment  $\times$  stimulus treatment:  $z = -1.970$ ,  $p = 0.0488$ ; electronic supplementary material, figure S2b,c). On trial 1, bees performed better when they were trained to purple/linalool flowers (trial  $\times$  stimulus treatment:  $z = -2.454$ ,  $p = 0.0141$ , stimulus treatment:  $z = 9.444$ ;  $p = 0.00421$ ). In line with previous findings [21], imidacloprid also impaired bees' motivation to forage, and their ability to collect sucrose from flowers (electronic supplementary material).

### (b) Test trial performance

Imidacloprid-treated bees made errors indicating impairment to olfactory, but not visual, learning. Specifically, these bees

	stimuli bees trained to	test stimuli			
		CS+ colour CS+ scent	CS- colour CS- scent 'both incorrect'	CS+ colour CS- scent 'scent error'	CS- colour CS+ scent 'colour error'
blue/geraniol CS+ ( <i>n</i> = 16)	G <sup>+</sup> L <sup>-</sup>	G	L	L	G
purple/linalool CS+ ( <i>n</i> = 17)	L <sup>+</sup> G <sup>-</sup>	L	G	G	L

**Figure 1.** The training and test conditioned stimuli (CS) (circle colour indicates flower colour; G = geraniol-scented, L = linalool-scented; + = rewarding, - = unrewarding). Sample size indicates the bees that completed both training trials and performed in the test trial.



**Figure 2.** (a) Number of visits (of the total of 20 flower visits) to the three incorrect flower types by treatment; (b) what differences between the pesticide versus control treatments would indicate; terminology used for the error type as it is referred to in the main text. Asterisk indicates significance at a level of  $p < 0.05$ .

made more scent errors than control bees (error type  $\times$  pesticide treatment:  $z = 2.302$ ,  $p = 0.0213$ ; pesticide treatment:  $z = -2.280$ ,  $p = 0.023$ ; figure 2a; electronic supplementary material, figure S3a), regardless of the stimulus combination they were trained to (Tukey *post hoc* tests: imidacloprid-treated bees made more scent errors both for blue/geraniol-trained bees:  $z = -5.659$ ,  $p < 0.0001$ , and for purple/linalool-trained bees:  $z = -2.983$ ,  $p = 0.0029$ ). While both imidacloprid-treated and control bees made more colour than scent errors overall (error type:  $z = 3.847$ ,  $p = 0.00012$ ; figure 2a), they did not differ in the number of colour errors they made (Tukey *post hoc* comparison:  $z = -0.672$ ,  $p = 0.502$ ; figure 2a; electronic supplementary material, figure S3a). Overall, imidacloprid-treated bees did not differ from controls in the number of correct flower choices they made in the test (pesticide treatment:  $z = 1.063$ ,  $p = 0.288$ ; stimulus treatment:  $z = 0.573$ ,  $p = 0.567$ ). Regardless of pesticide treatment, bees that were trained to the purple/linalool stimulus made more scent errors than bees trained to blue/geraniol (error type  $\times$  stimulus treatment:  $z = -2.650$ ,  $p = 0.00804$ ; electronic supplementary material, figure S3b). Although we cannot be certain because colour and scents were always paired in the current study, this result may be because purple was preferred over blue (supported by a previous finding with colour only [21] and consistent with our finding that

81% of bees went to purple/linalool flowers first on trial 1). Therefore, bees were more likely to visit purple test flowers even when they contained the incorrect scent.

## 4. Discussion

A growing body of research demonstrates that neonicotinoid pesticides have negative effects on bees [36], leading to recent restrictions in the EU [37]. In addition to impaired colony reproduction [19], neonicotinoids are associated with a number of sub-lethal behavioural effects [19], including impacts on bees' ability to learn floral stimuli [14]. A missing piece of this emerging picture is the extent to which these impairments, documented nearly exclusively in relation to olfactory learning, transfer when bees encounter more sensorially realistic flowers and naturalistic foraging scenarios. We offered free-flying bumblebees flowers that varied in scent and colour, and found that olfactory learning, but not visual learning was impaired by acute exposure to the neonicotinoid imidacloprid: exposed bees made more errors visiting flowers of the incorrect scent than controls, for both combinations of stimuli that individuals were trained to. These results explain at least some of the discrepancies in previous findings, and raise further questions about the mechanism behind olfactory learning impairment, which could have implications for how these pesticides affect multiple aspects of bees' ecology.

How might neonicotinoids differentially impair olfactory learning? Neonicotinoids affect the insect brain by acting as agonists of nicotinic acetylcholine receptors (nAChRs), with different neonicotinoids likely differentially activating specific receptor subtypes [38–40]. It is possible that nAChR subtype expression differs in the olfactory and visual systems, leading to the differential effects of imidacloprid exposure observed here. In the honeybee, imidacloprid can impair odour coding in antennal lobes, the first odour processing centre of the bee brain [41], and may even impair detection of volatiles upstream in antennal sensilla through action on odorant-binding proteins [42]. To our knowledge, the effects of imidacloprid on the bee visual system at an analogous peripheral or central level have not been determined (but see [43]).

Beyond their effects on single sensory modalities, neonicotinoids also impair other aspects of cognition, e.g. spatial working memory [34] and navigation [44]. These effects may be driven by neonicotinoids activating nAChRs in the Kenyon cells of the mushroom bodies [45,46], structures associated with cognition [47,48]. Given that these structures

are also involved in multisensory integration [23], we might expect this also to be impaired by neonicotinoid exposure. While we did not find such evidence in the current study, this could be a useful avenue for future research.

Another clear result was that neonicotinoid-exposed bees were less motivated to forage (as in [21,49,50]). A lack of foraging motivation may explain some of the learning impairments found in previous studies: most have addressed pesticide effects on learning using the PER protocol [30,31] (Fig. 1 in [21]). While this protocol has proved extremely useful for studying learning in bees [51], because bees are immobilized, identifying specific effects on foraging behaviour can be difficult. For example, effects on motivation may be confounded with effects on learning. In the current study, all bees exhibited PER for sucrose when stimulated at the start of trial 1, but many were not motivated to subsequently forage. If this had been assessed in a PER protocol these bees may have been included as being 'motivated' to exhibit PER (when stimulated) but may not have been sufficiently motivated to exhibit PER for a conditioned stimulus alone. Since this is the measure of learning in PER assays, motivational deficits could be misinterpreted as learning deficits.

What consequences might the impairment to olfactory learning we found here have for bee performance more generally? Crucially, olfactory impairment was not always compensated for by the availability of visual cues: when blue/geraniol flowers were rewarding, imidacloprid-treated bees performed worse than control bees during the learning trials. That this effect was driven by the olfactory rather than the visual cue is supported both by the test results and by previous work where colour learning was unaffected by imidacloprid across a range of doses [21]. The lack of an effect during training for purple/linalool-trained bees is likely because this was the preferred flower type, and thus it might be more difficult to impair learning in relation to this stimulus. If not only learning, but also olfactory

perception more generally is impaired [41,42], then neonicotinoids may have more serious holistic effects on behaviour than previously recognized. Bees rely upon chemoreception for a variety of foraging and non-foraging tasks [52], including pollen detection [53], nest recognition [54] and pheromonal communication within the colony [55], and thus any or all of these behaviours could also be disrupted.

While we did not find impairment to visual associative learning here, it is possible that it could be impaired under different scenarios, e.g. involving different neonicotinoids, chronic rather than acute exposure or when flowers offer different scents or colours. In the current study, the colour stimuli we chose were relatively difficult for bees to learn to discriminate compared with the scent stimuli, so if our effects simply reflected differences in stimulus discriminability, we would predict the opposite result (i.e. greater impairment to colour learning). However, a clear next step would be to test our finding over a broader range of stimuli. Given that generalist bees rely on a broad range of cognitive abilities [56], moving towards assays that measure cognition in more naturalistic settings is critical to fully understand these pesticides' effects on bees.

**Data accessibility.** Data available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.87p3dd7> [57].

**Authors' contributions.** All authors contributed to the design of the experiment. F.M. collected the data, carried out the statistical analyses and wrote the initial version of the manuscript. All authors agree to be held accountable for the content and approve the final version of the manuscript.

**Competing interests.** We have no competing interests.

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