

RESEARCH ARTICLE

A novel protocol for studying bee cognition in the wild

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Abstract

1. Understanding how animals perceive, learn and remember stimuli is critical for understanding both how cognition is shaped by natural selection, and how ecological factors impact behaviour. However, the majority of studies on cognition involve captive animals in laboratory settings. While controlled settings are required to accurately measure aspects of cognition, they may not yield realistic estimates of learning performance in natural environments. Wild bees offer a useful system in which to study cognitive ecology and comparative cognition more broadly: they encompass around 20,000 species globally, varying in characteristics such as life-history strategy, degree of sociality and dietary specialization. Yet, the limited number of protocols currently available for studying insect cognition has restricted research to a few commercially available bee species, in almost exclusively laboratory settings.
2. We present a protocol (Free-Moving Proboscis Extension Response [FMPER]) to measure wild bees' colour preferences, learning performance and memory.
3. We first used laboratory-reared bumblebees *Bombus impatiens* to establish that FMPER yielded results consistent with learning theory. We then successfully tested wild honeybees *Apis mellifera* in the laboratory and *Bombus vosnesenskii* at field sites.
4. Free-Moving Proboscis Extension Response is straightforward to implement, is low cost, and may be readily adapted to other flower-visiting insects. We believe it will be useful to a broad range of evolutionary biologists, behavioural ecologists and pollination ecologists interested in measuring cognitive performance in the wild and across a broader range of species.

KEYWORDS*Apis*, behaviour, *Bombus*, bumblebee, cognition, field, honeybee

1 | INTRODUCTION

Cognition encompasses the mechanisms by which animals acquire, process, store and act on information (Shettleworth, 2010). Like morphological and physiological traits, cognitive abilities contribute to how effectively animals perform in their environments (e.g. foraging for resources, selecting mates and rearing offspring). However, cognitive traits can be problematic to quantify in field populations. Due to numerous confounding variables, carefully designed experiments are

needed to measure interspecific or intraspecific variation in cognitive performance (Morand-Ferron, Hamblin, Cole, Aplin, & Quinn, 2015; Rowe & Healy, 2014). Perhaps because of this, study of cognition in the wild has historically been restricted to observational studies, with the vast majority of experiments involving either laboratory-reared animals or wild animals brought into captivity.

Cognition is shaped both by natural selection and by experiences during an individual's lifetime (Dukas, 1998). As such, laboratory-based studies with captive-bred animals often demonstrate what an

animal *can* do, rather than what it actually *does* in its natural environment. To gain an ecologically relevant measure of cognitive performance (Pritchard, Hurly, Tello-Ramos, & Healy, 2016), researchers have thus taken methods from the psychology laboratory into the field (Morand-Ferron, Cole, & Quinn, 2016), addressing topics such as spatial memory in chickadees and hummingbirds (Croston et al., 2016; Flores-Abreu, Hurly, & Healy, 2013), associative learning in great tits (Morand-Ferron et al., 2015) and tool use in chimpanzees (Biro et al., 2003). However, despite progress in field-based cognitive ecology of vertebrate systems, invertebrate cognition is still largely tested in laboratory-reared animals under laboratory conditions (but see Collett, Chittka, & Collett, 2013).

Eusocial bees comprise a major system for the study of learning and its ecological consequences. As dietary generalists, *Apis* and *Bombus* foragers visit flowers that vary in their floral display and reward composition, and rapidly learn associations between floral features and rewards. However, both individuals (Scheiner, Page, & Erber, 2001) and colonies (Ings, Raine, & Chittka, 2009; Raine & Chittka, 2007) vary in their colour preferences and learning abilities, with implications for colony-level fitness (Raine & Chittka, 2007, 2008). Foragers' learning performance also has clear effects on the fitness of their mutualistic partners: imperfect learning may lead to pollen being transferred among different species (Leonard & Masek, 2014) at a reproductive cost to plants (Arceo-Gómez & Ashman, 2011).

The majority of modern research on bee cognition occurs in a laboratory setting (Giurfa, 2007) on three commercially available social species: the honeybee *Apis mellifera* and the bumblebees *Bombus impatiens* or *Bombus terrestris*. Given that bee species show species- and population-level variation in correlates of interest to comparative cognition researchers (e.g. degree of sociality, dietary specialization, life span and reproductive strategy) and inhabit environments varying in aspects of interest to cognitive ecologists (e.g. floral diversity and availability), there are surprisingly few comparative studies of bee learning. The small number that exist hint at patterns that deserve further investigation: learning performance varies between a social (*Bombus bimaculatus*) and solitary (*Xylocopa virginica*) species (Dukas & Real, 1991; see also Mc Cabe, Hartfelder, Santana, & Farina, 2007; Moreno, de Souza, & Reinhard, 2012), between Africanized and European honeybees (Couvillon, DeGrandi-Hoffman, & Gronenberg, 2010) and between European and dwarf honeybees (Kaspi & Shafir, 2013).

Methods of investigating bee cognition typically involve free-moving assays where bees fly or walk between flowers (Russell, Golden, Leonard, & Papaj, 2016) or floral surrogates (Muth, Papaj, & Leonard, 2015). Over the course of training, individuals learn to associate a "conditioned stimulus" (CS; a stimulus to which an individual does not have a prior response, e.g. a colour) with an "unconditioned stimulus" (US; a stimulus to which naive individuals respond, e.g. nectar, which reflexively leads to proboscis extension). These free-moving protocols are not tractable for wild bees, as they often require extensive pre-training to a floral array, and it can be difficult to keep social bees motivated for long periods while away from their colony. Free-moving methods are also often time consuming because bees must be trained individually to minimize social influences (Leadbeater &

Chittka, 2007). This limits the rate of data collection, yielding small sample sizes (10–20 bees per treatment group is not uncommon). Further, in free-moving assays, the individual bee controls the order and timing of stimulus presentation (when and which flowers it visits), which adds noise to individual performance comparisons.

The second main protocol used to study cognition in insects is the "Proboscis Extension Response (PER) protocol" (honeybees: Bitterman, Menzel, Fietz, & Schäfer, 1983; Takeda, 1961; bumblebees: Laloi et al., 1999; Riveros & Gronenberg, 2009; fruitflies: Tully & Quinn, 1985; mosquitoes: Tomberlin, Rains, Allan, Sanford, & Lewis, 2006; moths: Daly & Smith, 2000; stingless bees: Mc Cabe et al., 2007; solitary bees: Kapheim & Johnson, 2017; hymenopterous parasitoids: Kaiser, Pérez-Maluf, Sandoz, & Pham-Delègue, 2003). In the PER protocol, a bee is harnessed (only its antennae and proboscis free to move [Figure 1b]), before being trained to an association between a CS and US. If the individual learns the association, it will extend its proboscis when the CS (e.g. a scent) is presented. The PER protocol has proved extremely useful in testing many aspects of bee learning, including the effects of nectar chemistry (Wright et al., 2013), stress (Muth, Scampini, & Leonard, 2015) and pesticides (Stanley, Smith, & Raine, 2015; Williamson & Wright, 2013). It offers several advantages over free-moving assays: the timing of stimulus presentation can be tightly controlled and individuals can be trained in parallel (in our experience, ~10–12 bees per day). However, despite the advantages of this technique, harnessed bees often behave differently to free-moving bees, for example, accepting different concentrations of sucrose (Mujagic & Erber, 2009), and being more likely to ingest toxic substances (Ayestaran, Giurfa, & de Brito Sanchez, 2010). We have also found that *B. impatiens* are more responsive to sucrose when free moving than when harnessed (Muth et al., in prep).

Here, we present a novel learning protocol, Free-Moving Proboscis Extension Response (FMPER), to test ecologically relevant measures of cognition in captive and wild bees. Free-Moving Proboscis Extension Response allows researchers to measure aspects of cognition similar to those tested in harnessed PER protocols (e.g. responsiveness, learning and memory). At the same time, it likely avoids the negative effects of harnessing and is non-lethal. We used FMPER to study associative learning and memory in relation to sucrose rewards. From this, we argue that a field-based behavioural assay has the potential to unlock many new lines of research, among them quantification of cognitive or sensory traits at the population or species level, about which very little is known regarding wild bees or invertebrates more generally.

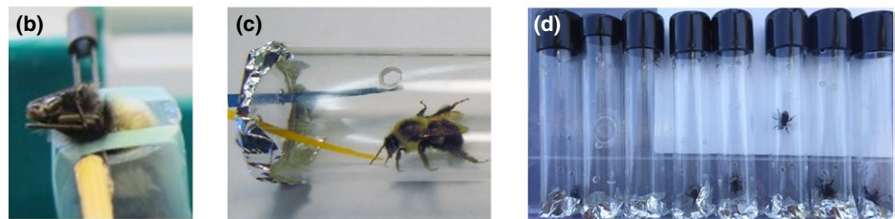
To test whether FMPER yields data matching the predictions of learning theory, we initially trialled it in the laboratory using commercially sourced bumblebees (*B. impatiens*). We investigated colour preferences, absolute conditioning (learning to respond to a single conditioned stimulus), differential conditioning (learning to discriminate between two conditioned stimuli) and memory. Having established that laboratory-reared bees learned in the FMPER protocol, we then tested wild-caught *A. mellifera* brought into a laboratory setting and wild *Bombus vosnesenskii* in situ at field sites (summary in Figure 1a).

In all contexts, FMPER generated sample sizes in hours that would take days or weeks to acquire in a free-flying experiment. Because it

(a)

	Does the method work under lab conditions?		Does the method work in a different bee genus?	Does the method work under field conditions?	
Experiment	1a	1b	2	3a	3b
Species used	<i>Bombus impatiens</i>		<i>Apis mellifera</i>	<i>Bombus vosnesenskii</i>	
Method used	Single-choice	Multi-choice	Single-choice	Single-choice	Multi-choice
Lab vs. wild	Lab reared, tested in lab		Field-caught, tested in lab	Field-caught, tested <i>in situ</i>	

FIGURE 1 (a) Summary of all experiments with focal question at the top; (b) a bee harnessed in the traditional PER protocol; (c) the FMPEP protocol: a bee (*Bombus impatiens*) is presented with two stimuli (blue and yellow strips) and approaches the yellow strip to exhibit PER; (d) eight wild-caught *Bombus vosnesenskii* being trained at a field site



does not require previous experience conducting learning experiments or maintaining bees in captivity, FMPEP will allow a wider range of researchers to obtain ecologically relevant measures of cognitive abilities, and to ask questions about the cognitive ecology of pollination and comparative cognition that push these fields in new directions.

2 | MATERIALS AND METHODS

2.1 | FMPEP apparatus and protocols

In all experiments, we collected foragers on either artificial or real flowers using a net (in the field) or customized aspirator (in the laboratory: Bioquip Products, item #2820GA). Different individuals were used in all experiments, and all bees were only ever tested once. We then transferred bees to transparent plastic cylindrical tubes (TAP plastics, USA) with ventilation holes (L x D 13 x 2.5 cm, wall thickness: 1.6 mm) (Figure 1c). The tube was sealed at one end by a size 4 rubber stopper (to prevent escape), and at the other by a transparent plastic disc (with two 3 mm diameter holes) affixed with metal tape (Figure 1c). This disc served as the location for training and testing. The bee was allowed to acclimatize to the tube for 1 hr (*A. mellifera*) or 2 hr (*Bombus*); pilot work established that after these periods, bees were motivated to participate in the experiment (for details, see Supplementary Methods).

We used strips of coloured card (W x L 1.0 x 20 mm) to test bees' colour preferences, learning and memory. These coloured stimuli (human-blue, orange, green, yellow "1" and yellow "2") were plotted into a model of bee colour space which uses the reflectance spectra of the card, the irradiance, and the bees' spectral sensitivities to estimate the difficulty of a discrimination task (Chittka, 1992) (Supplementary Material, Figure S1). During training, each strip was dipped into a solution (either a positively reinforcing US (US+): 50% [w/w] sucrose, or an unrewarding (water) or negatively reinforcing US (US-): 5% [w/w]

NaCl or quinine solution), resulting in ~10 µl of solution on each strip. Once inserted 1 cm into the tube, we allowed bees to drink the US+ or US- from the strip before removing it. We waited until the bee was at the far end of the tube before insertion, so that it could see the strip before approaching it. In all treatments across experiments, half the bees were always trained to one colour and half to the other.

2.2 | Single-choice protocol

To test for *initial colour preferences*, we inserted two equally rewarding 50% (w/w) sucrose strips of different colours (positions counter-balanced across subjects to account for side biases) into each of the disc holes. We recorded the first colour a bee extended its proboscis towards.

For the *absolute conditioning procedure*, we gave each bee five presentations of a strip of a given colour offering 50% (w/w) sucrose, separated by 5-min intervals. Five minutes after the final presentation, we presented bees with two colours (the colour previously rewarded and a novel colour) in an unrewarded test phase (both strips offered water), and recorded which colour the bee exhibited PER towards first. The position of the positively reinforcing conditioned stimulus (CS+) was alternated between the two disc holes during training and randomized across subjects.

For the *differential conditioning procedure*, we carried out five trials. On each trial, we presented bees with the CS+ followed by the CS- (a negatively reinforcing stimulus such as water, a solution of 5% NaCl, or a solution of 5% quinine, depending on the experiment). We always presented the CS+ first, allowing the bee to drink from it for 5 s (Experiment 1, 2) or 3 s (Experiment 3). We then removed the CS+ and then presented the CS- and allowed the bee to drink from it for 5 s (Experiment 1, 2) or 3 s (Experiment 3). Five minutes after the final presentation, we presented bees with two colours in an unrewarded test phase as described above. Trials and test were spaced 5 min apart and the position of the CS+ was alternated as described above.

2.3 | Multi-choice protocol

The single-choice protocol resulted in one data point per bee (whether the bee learned or not), but performance over multiple trials yields more data per individual and allows comparison of differences in acquisition (learning curve slopes). To generate learning curves, we trained bees (captive *B. impatiens*: Experiment 1b; wild *B. vosnesenskii*: Experiment 3b) using a modified protocol where learning was assessed over a series of choices as well as in a final test trial. In this protocol, we gave bees two trials using differential conditioning (described above), followed by a further seven trials (10 min apart) where we recorded the bee's choice between the CS+ and CS- presented simultaneously. After the bee extended its proboscis to a strip and sampled the CS+ or CS- for 3 s, that strip was removed and the bee was given the other strip for 3 s. Thus, all bees received both the CS+ and the CS- on each trial, but the order of presentation was determined by the bee. We made sure that the bee did not antennate strips before extending its proboscis to ensure that the decision was not based on gustatory cues.

In all learning experiments, the test (or "probe" trial) established whether bees had learned the association with the CS and were not responding to an additional cue from the US. Accordingly, no rewards were used in the test phase (water only).

For both protocols, we suggest means by which the data can be analysed (Supplementary Material).

2.4 | Experiment 1a: Captive bees tested in the single-choice FMPEP protocol

We tested captive bumblebees (*B. impatiens*) (for methodological details, see Supplementary Material) using the single-choice FMPEP protocol to determine the conditions under which learning would occur, and whether it followed the general rules of learning theory. To do this, we initially tested bees for colour preferences (protocol described above), before training bees either via absolute or differential conditioning.

We used two colour comparisons: orange vs. green or blue vs. yellow 1, to ensure that our findings held across multiple colour comparisons that were roughly equivalent: visual modelling allowed us to calculate the chromatic contrast [CC] of strip colours, which suggested that blue vs. yellow 1 discrimination (CC = 0.201), should be only marginally more difficult than orange vs. green discrimination (CC = 0.159; colours plotted in bee colour space in Figure S1), supported by previous findings in a related species (Chittka, 1992; Dyer, Spaethe, & Prack, 2008).

We tested bees via absolute and differential conditioning (following the procedure outlined above) to determine whether, in line with learning theory, bees would learn faster when trained via differential conditioning. We used two different negatively reinforcing stimuli (5% NaCl solution and 5% quinine solution) because we wanted to compare FMPEP results to the previous finding that NaCl is more aversive than quinine to harness, but not free-moving, bees (de Brito Sanchez, Serre, Avargues-Weber, Dyer, & Giurfa, 2015).

To see all treatment groups and sample sizes, see Table S1.

2.5 | Experiment 1b: Captive bees tested in the multi-choice FMPEP protocol

The results of Experiment 1a demonstrated that bees could learn under the single-choice FMPEP protocol, but also suggested that differences between treatments might be difficult to detect if learning performance is condensed to a single (test) outcome. We thus developed a multi-choice FMPEP protocol suitable for generating acquisition curves. To trial this protocol, we assigned captive *B. impatiens* to one of four treatment groups (Table 1). Within each of these treatments, approximately half of the bees were trained to one colour, and half were trained to the other.

We predicted that bees would learn more readily when the US was more aversive (quinine vs. water), thus treatments 1 and 3 would outperform treatments 2 and 4, respectively. We also predicted that learning would be more difficult when CSs were harder to discriminate (yellow 1 vs. yellow 2, compared with yellow 1 vs. blue), thus treatments 1 and 2 would outperform treatments 3 and 4, respectively.

2.6 | Experiment 2: Wild-caught honeybees tested in the laboratory

We caught 140 honeybees *A. mellifera* (September–October 2016) foraging on the campus of the University of Nevada, Reno (NV, USA). We tested individuals on their initial colour preferences and learning performance (via absolute and differential conditioning) following the same single-choice FMPEP protocol as Experiment 1a (for treatments and sample sizes, see Figure 4).

2.7 | Experiment 3a: Wild bumblebees learning in situ: single-choice FMPEP

In June 2016 as part of a larger study, we caught 40 *B. vosnesenskii* females from flowers in Mayberry Park, Reno (NV, USA), and

TABLE 1 Treatments in experiment 1b. This experiment was designed to test whether findings from the FMPEP assay would agree with learning theory, specifically whether bees would learn more easily when the negatively reinforcing unconditioned stimulus was more aversive and where conditioned stimuli were easier to discriminate. Chromatic contrast (CC) indicates how discriminable the colours were to the bee and correlates with task difficulty

	CS- (CS+ always 50% sucrose)	
	More aversive (quinine) Faster learning predicted	Less aversive (water) Slower learning predicted
Colour discrimination		
Easy (CC: 0.201) Faster learning predicted	Treatment 1: n = 20	Treatment 2: n = 26
Difficult (CC: 0.032) Slower learning predicted	Treatment 3: n = 18	Treatment 4: n = 20

trained them via differential conditioning using the single-choice FMPEP protocol (blue vs. yellow 1, $n = 20$ trained to each colour; US+ = 50% (w/w) sucrose, US- = water; [Figure 1d]). We carried out trials in a shaded area <1.5 km from where bees were collected. To prevent recapture of bees and for genetic analyses as part of a larger study, we clipped their left midleg tarsus before release.

2.8 | Experiment 3b: Wild bumblebees learning in situ: multi-choice FMPEP

Using the multi-choice FMPEP protocol, we trained 61 *B. vosnesenskii* females (~24 bees per day) to a blue vs. yellow discrimination task ($n = 30$ trained to blue, $n = 31$ trained to yellow) between July and August 2016 at a site near Sagehen Creek (CA, USA). As in Experiment 3a, we clipped the midleg tarsus of all bees and fed all bees to satiation before releasing them.

2.9 | Data analyses

All analyses were carried out in R v 3.2.5. For mixed models, we used the `glmer()` function in the `LME4` package (Bates, Mächler, Bolker, & Walker, 2015). In all final models, we included all the variables that we manipulated as part of the experimental design. To determine whether interaction terms should be included, we carried out maximal models initially, and then decided whether to include interaction terms through selecting the most parsimonious model using AIC values. Information on the analyses used in each experiment is given in the Supplementary Material and is summarized in the tables in Results section.

3 | RESULTS

3.1 | Experiment 1a: Captive bees tested in the single-choice FMPEP protocol

Bombus impatiens did not initially prefer any of the test colours, but did readily learn associations between colour and a sucrose reward in all cases. However, whether bees were trained via absolute or differential conditioning did not affect test performance, nor did whether NaCl or quinine solution was used as the US-. Bees could readily recall learned associations when tested 30 min after training. For results, see Table 2 and Figure 2.

3.2 | Experiment 1b: Captive bees tested in the multi-choice FMPEP protocol

Bees differed in their learning performance in line with predictions: bees that received quinine as the US- performed better than bees that received water as the US-, and there was a strong trend for bees trained to the putatively easier (blue vs. yellow 1) discrimination to learn the association better than bees trained to the more difficult discrimination (Table 3, Figure 3). Surprisingly, bees did not generally improve in their performance across successive trials. This makes sense for the two quinine US- groups (treatments 1 and 3): after receiving the two presentations of the paired CS-US stimuli prior to the first choice trial, their performance was already at ~90% correct, leaving little room for improvement. However, the two other treatments trained with water as the US- (treatments 2 and 4) also did not improve over successive trials: their above-chance performance was established in the first two presentations. All interaction terms were non-significant (Table S2).

In the test phase, all treatments performed above chance, indicating that they learned, with more bees choosing correctly than incorrectly (χ^2 tests: treatment 1: $\chi^2_1 = 20$; $p < .0001$; treatment 2: $\chi^2_1 = 14.44$;

TABLE 2 Experiment 1a results. Results are from models that include all fixed effects. Interaction terms were not included since they resulted in the model being less parsimonious (see “Data Analyses” section of Supplementary Material for details)

Question	Colour comparison	Analysis	Model	Test statistic and degrees of freedom	p-value
Did bees prefer one colour over the other?	Blue vs. yellow	χ^2 test		$\chi^2_2 = 3.200$.074
	Orange vs. green	χ^2 test		$\chi^2_2 = 0.2$.655
Were bees more likely to choose the colour that was previously rewarding? (i.e. learn), and was this affected by training procedure?	Blue vs. yellow	Binomial GLM	Choice (blue or yellow) =		
			Colour trained to (blue or yellow)	$z_{79} = -4.568$	<.0001
			+ training procedure (absolute or differential)	$z_{79} = 1.627$.104
	Orange vs. green	Binomial GLM	Choice (blue or yellow) =		
			Colour trained to (blue or yellow)	$z_{77} = -4.071$	<.0001
			+ training procedure (absolute or differential)	$z_{77} = 0.763$.445
Did learning depend on whether NaCl or quinine was used as the US-?	Blue vs. yellow	Binomial GLM	Choice (blue or yellow) =		
			Colour trained to (blue or yellow)	$z_{77} = -5.517$	<.0001
			+ US- (NaCl or quinine)	$z_{77} = -0.624$.533
Did bees remember associations long term?	Blue vs. yellow	Binomial GLM	Choice (blue or yellow) =		
			Colour trained to (blue or yellow)	$z_{39} = -4.097$	<.0001

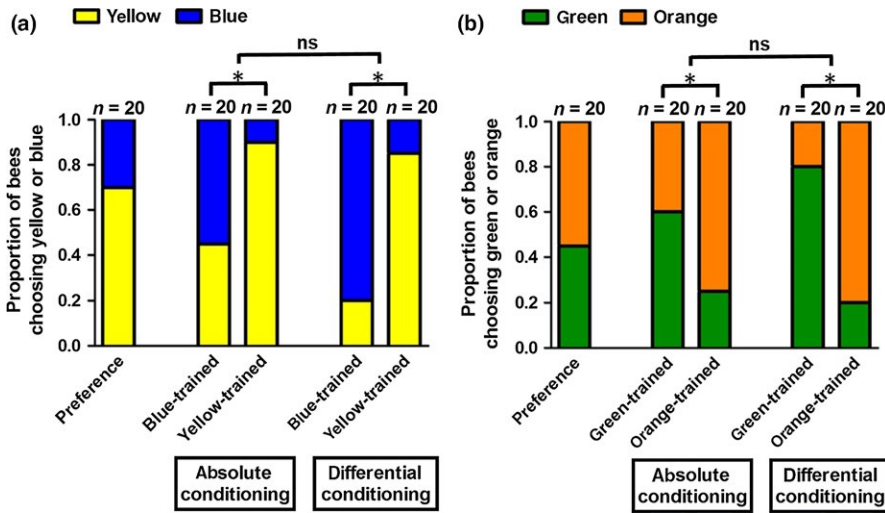


FIGURE 2 The proportion of commercial, laboratory-reared *Bombus impatiens* that chose: (a) yellow 1 or blue; or (b) green or orange: in their initial preference test, after absolute conditioning (50% sucrose), and after differential conditioning (50% sucrose vs. 5% NaCl; quinine and long-term data not shown here). Asterisk: statistical significance (ns = non-significant); see Table 2

TABLE 3 Experiment 1b results. Results are from models that include all fixed effects. Interaction terms were not included since they resulted in the model being less parsimonious (see “Data Analyses” section of Supplementary Material for details)

Question	Analysis	Model	Test statistic	p-value
Did bees make more correct choices across successive trials, and was this affected by the discriminability of stimuli and the aversiveness of the CS-?	Binomial GLMM	Correct/incorrect (1/0) = trial (1–7)	$z = 1.204$.229
		+ colour treatment (“easy” or “difficult”)	$z = -1.913$.056
		+ CS- (water or quinine)	$z = -5.743$	<.0001
Did the discriminability of stimuli and the aversiveness of the CS- affect test performance?	Binomial GLM	Correct/incorrect (1/0) =		
		+ colour treatment (“easy” or “difficult”)	$z_{81} = -0.613$.540
		+ CS- (water or quinine)	$z_{81} = -1.644$.100

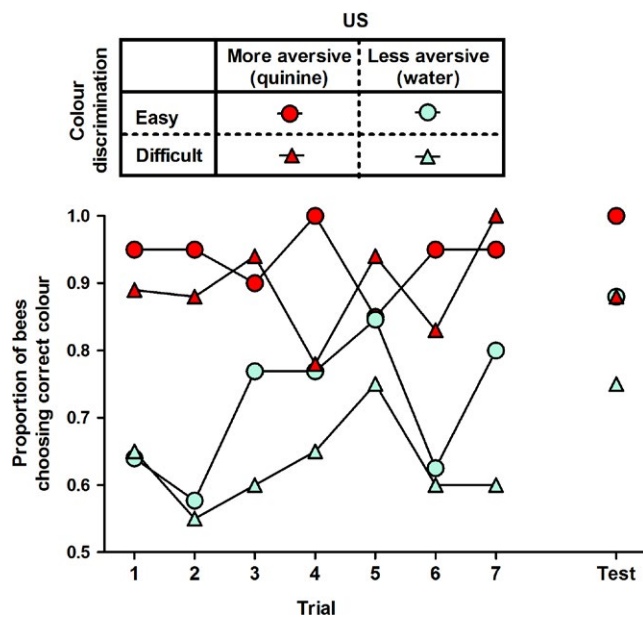


FIGURE 3 Performance of bees across successive trials and in the test phase using the multi-choice FMPER protocol. Triangles: more difficult discrimination task (blue vs. yellow); circles: easier discrimination task (yellow 1 vs. yellow 2). Red: quinine as the unconditioned stimulus (US); blue: water as the US. Statistical results: Table 3

$p < .0001$; treatment 3: $\chi^2_1 = 3$; 9.94; $p < .01$; treatment 4: $\chi^2_1 = 5$; $p < .05$). However, the aversiveness of the US- and discriminability of stimuli did not generally affect test performance (Table 3, Figure 3),

except in the most extreme comparison: bees trained on the “easy” discrimination with quinine as US- outperformed bees trained on the “difficult” discrimination with water as the US- ($\chi^2_1 = 5.714$; $p < .05$).

3.3 | Experiment 2: Wild-caught honeybees tested in the laboratory

Apis mellifera trended in the direction of choosing more strips of the correct colour when considering the data on absolute and differential conditioning with water. Here, the training procedure did not significantly affect learning performance. When addressing only the data where bees were trained via differential conditioning, bees readily learned, and the US used did not affect this. To determine whether bees learned within each treatment, we carried out χ^2 tests: bees trained via absolute conditioning did not learn, but bees in the two differential conditioning treatments did (χ^2 tests: absolute conditioning: $\chi^2_1 = 0.10526$; $p = .746$; differential conditioning with water: $\chi^2_1 = 4.9$; $p < .05$; differential conditioning with 5% NaCl: $\chi^2_1 = 8.805$; $p < .005$). Individuals did not initially prefer any of the test colours. For all results, see Table 4 and Figure 4.

3.4 | Experiment 3a: Wild bumblebees learning in situ: single-choice FMPER

Wild *B. vosnesenskii* learned readily in the single-choice FMPER protocol, with bees trained to blue strips being more likely to choose them in the test than bees trained to yellow ($df = 38$; $z = -3.265$; $p < .005$; Figure 5a).

TABLE 4 Experiment 2 results. Results are from models that include all fixed effects. Interaction terms were not included since they resulted in the model being less parsimonious (see “Data Analyses” section of Supplementary Material for details)

Question	Analysis	Model	Test statistic and degrees of freedom	p-value
Did bees prefer one colour over the other?	χ^2 test		$\chi^2_2 = 0.200$.655
Were bees more likely to choose the colour that was previously rewarding? (i.e. learn), and was this affected by training procedure?	Binomial GLM	Choice (blue or yellow) = Colour trained to (blue or yellow) + training procedure (absolute or differential)	$z_{79} = -1.546$ $z_{79} = -0.986$.065 .324
Did bees that were trained via differential conditioning learn and was this affected by the unconditioned stimulus used?	Binomial GLM	Choice (blue or yellow) = colour trained to (blue or yellow) + US (water or quinine)	$z_{81} = -3.559$ $z_{81} = -0.077$	<.0001 .938

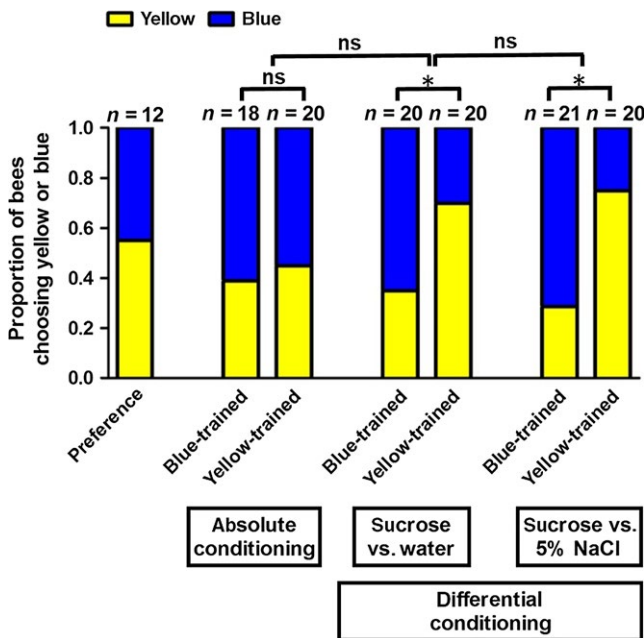


FIGURE 4 The proportion of wild-caught *Apis mellifera* that chose yellow or blue: in initial preference tests, after absolute conditioning (50% sucrose), and after differential conditioning (50% sucrose vs. water or 50% sucrose vs. 5% NaCl). Asterisk: statistical significance (ns = non-significant); see Table 4

3.5 | Experiment 3b: Wild bumblebees learning in situ: multi-choice FMPER

Bombus vosnesenskii tested using multi-choice FMPER made more correct choices over successive trials, unaffected by the colour they had been trained to. Bees were also more likely to choose the colour they had been trained to in the test phase (Table 5; Figure 5b).

4 | DISCUSSION

Cognition shapes interactions between plants and floral visitors, as the efficacy with which pollinators learn and remember floral stimuli

contributes to the relative fitness of both parties. Thus, comparison of pollinator learning at individual, population, and species levels represents the opportunity to study the causes and consequences of variation in cognitive performance. To date, however, the majority of research has focused on a handful of bee species, confined to laboratory settings and involves commercial lineages that may have been subject to quite different selection pressures (perhaps even relaxed selection for learning). Here, we demonstrated a new protocol that can be used to measure bee cognition in the field, and thus be adapted to explore how ecological variables influence sensory abilities, preferences, learning and memory in wild populations. These measures are ecologically relevant to bees and plants, because they govern floral choices, rewards gained, and ultimately, colony fitness (Raine & Chittka, 2007, 2008).

From our laboratory-based trials with *Bombus* and *Apis*, we found that the single-choice FMPER protocol (Experiments 1a, 2 and 3a) usually resulted in bees learning. In line with learning theory (Shettleworth, 2010) and previous findings (Dyer & Chittka, 2004), differential conditioning led to better performance than absolute conditioning in *A. mellifera* (Experiment 2). While absolute conditioning may be sufficient for some questions, differential conditioning is necessary to guarantee a significant proportion of bees learn in five trials. Likewise, Experiment 1 showed that the single-choice FMPER procedure did not detect fine-scale learning differences among laboratory-raised *B. impatiens*: thus, this simpler protocol may be most useful for determining whether discrimination between two given colours is possible rather than comparing relative performance among treatment groups. Indeed, we would have needed a sample size of 159 subjects in each treatment (absolute vs. differential) to detect a significant difference at an alpha level of 0.05.

Thus, we developed the multi-choice FMPER protocol where we measured the choices of bees over a series of trials, resulting in group-level learning curves, and fewer individuals required to achieve sufficient power. Our laboratory-based trials with *B. impatiens* demonstrated that this protocol revealed the performance differences predicted by learning theory: subjects performed better when the US was more aversive and when the CSs were easier to distinguish. While captive *B. impatiens* performed above chance across trials (i.e. learned),

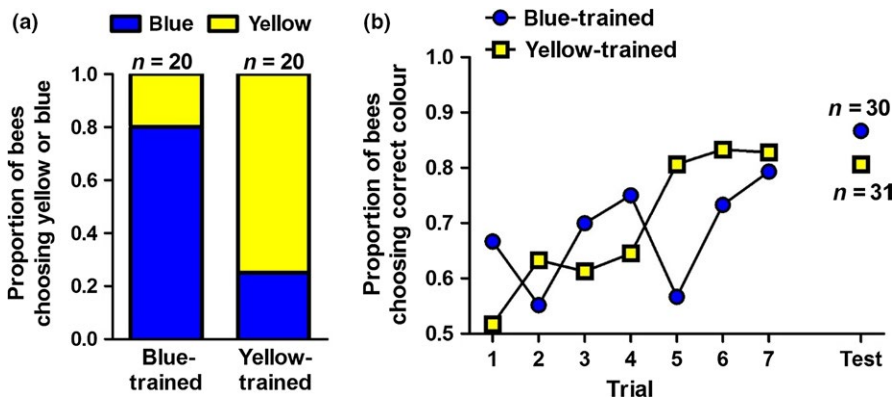


FIGURE 5 The choices of wild-caught *Bombus vosnesenskii* for blue or yellow strips when differentially conditioned using the (a) single-choice or (b) multi-choice FMPER protocol. For statistics, see Table 5

TABLE 5 Experiment 3b results. Results are from models that include all fixed effects. Interaction terms were not included since they resulted in the model being less parsimonious (see “Data Analyses” section of Supplementary Material for details)

Question	Analysis	Model	Test statistic	p-value
Did bees make more correct choices across successive trials (i.e. learn)?	Binomial GLMM	Correct/incorrect (1/0) = trial (1–7) + colour trained to (blue or yellow) + random factor “bee”	$z = 3.398$ $z = 0.123$	<.0001 .902
Were bees more likely to choose the colour that was previously rewarding in the test phase?	Binomial GLM	Choice (blue or yellow) = Colour trained to (blue or yellow)	$z_{59} = -4.688$	<.0001

we did not find overall improvement from the first choice trial (i.e. the third exposure to stimuli) to the seventh. This contrasts with our findings from field-caught *B. vosnesenskii* that not only performed above chance by the end of training but also improved in their performance across trials. This may reflect differences in sample size/variance, or perhaps wild bees were hungrier, accustomed to lower quality nectar, and/or further from their home colony: wild-caught bees appeared more motivated and less aggressive during training (F. Muth, personal observation). While our field-based results focused on *B. vosnesenskii*, we successfully trained a number of other species, including female *Bombus appositus*, *Bombus melanopygus*, *Bombus bifarius*, *Bombus vandykei* and *Bombus flavifrons*, and male *B. bifarius*, *Bombus flavidus* and *Bombus insularis*.

Free-Moving Proboscis Extension Response offers a number of advantages over other free-moving methods used to study insect behaviour: it allows the experimenter to control stimulus presentation, as well as the timing of reward presentation (an important variable in learning acquisition: Shettleworth, 2010). Further, FMPER can be run in parallel, resulting in ~10 bees trained in 30 min by one experimenter. It is non-lethal and could be readily modified for other nectivorous insects. Although we focused on visual learning, by customizing the strips or affixing petal tissue, FMPER could be adapted to measure learning of other stimuli, such as patterns, textures, temperatures, odours or multimodal combinations thereof (Leonard & Masek, 2014). Direct comparisons of, for example, visual vs. olfactory learning performance (e.g. Smith & Raine, 2014) would be relevant to establishing whether group-level differences in learning reflect a general processing ability or are specific to one modality (Spaethe, Brockmann, Halbig, & Tautz, 2007). In line with most work on bee cognition, the FMPER protocols we describe detect group-level differences. To gain more information

on individual performance differences, one could test bees over a greater number of choices (e.g. 50 choices, taking an average/10).

When taking FMPER into the field, there are potential pitfalls, associated with all studies of learning (Pritchard et al., 2016; Rowe & Healy, 2014). First, conditioning an individual to a colour that it already strongly prefers (Lunau & Maier, 1995) does not leave much room for learning. Thus, ideally stimuli would be used that individuals do not have strong preference for (established a priori as in Experiment 1). It may also be useful to collect data on the colours of flowers bees were foraging on, to control (to some extent) for recent foraging experience, known to affect colour preferences (Dyer & Chittka, 2004; Muth et al., 2015). It is also important to include at least two CS+ within each comparison group (e.g. counterbalancing bees trained to blue or yellow as the CS+). Without this, populations could appear to differ in their ability to learn a colour association, when in fact they simply differ in their preferences for the given CS+ (Raine & Chittka, 2007).

Motivation (determined by stress or hunger) can also affect learning rate, and should be controlled for as much as possible. We left all bees to sit prior to testing for times that were long enough to induce hunger, but not so long that the animals died. If differences in learning are found between groups, it may also be relevant to establish whether “non-cognitive” explanations exist (Barrett, 2014; Rowe & Healy, 2014). For example, bees may have differences in perceptual sensitivity (i.e. bees with larger eyes may be better able to learn visual associations) or in gustatory responsiveness (Scheiner et al., 2001). Ensuring that size and age (measured by wing-wear; Mueller & Wolf-Mueller, 1993) are equally represented across treatments could account for these possibilities.

Beyond enhancing the toolkit available to animal cognition researchers, FMPER holds broader potential for addressing questions relevant to ecologists and evolutionary biologists. For example, FMPER could

be used to explore cognitive differences between populations of wild bees exposed to stressors such as pesticides, parasites or poor nutrition, all implicated in their declines (Goulson, Nicholls, Botias, & Rotheray, 2015). It could also be used to investigate how secondary metabolites in nectar (Wright et al., 2013) affect wild bees' learning or nectar preferences. Using FMPER to measure how readily previously trained bees will learn a novel association, or switch their learned preference via reversal learning (Strang & Sherry, 2014) might speak to the ecological consequences of learning performance, given that bees that learn an association "better" may actually be worse at discovering new resources (Evans & Raine, 2014). Similarly, FMPER could be used to compare the floral constancy (Chittka, Thomson, & Waser, 1999) of floral visitors by sequentially presenting two equally rewarding conditioned stimuli. Or, field-based differential conditioning by FMPER might help ecologists investigate putative cases of floral mimicry, by establishing whether a bee can or cannot discriminate between floral stimuli of interest. Besides basic work on comparative cognition, FMPER's potential for assessing gustatory responsiveness (similar to Ma, Kessler, Simpson, & Wright, 2016) is relevant to many aspects of bee biology (Scheiner et al., 2001), including tests of hypotheses relating to the evolution of sociality (e.g. the reproductive ground plan hypothesis: Kapheim & Johnson, 2017).

The Free-Moving Proboscis Extension Response protocol can demonstrate differences in learning ability determined by properties of the conditioned stimulus (floral display) and unconditioned stimulus (here, nectar), but may also be used to reveal differences in bee cognition at the population or species level, driven by genetic differences and/or environmental factors. We believe this novel protocol will allow us to bring together pollinator ecology and cognition to ask exciting questions across a range of disciplines.

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AUTHORS' CONTRIBUTIONS

F.M., R.F.B. and T.R.C. collected the data. F.M. and A.S.L. analysed and interpreted data and wrote the manuscript. All authors contributed to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data deposited in the Dryad Digital Repository <https://doi.org/10.5061/dryad.c2k5t> (Muth, Cooper, Bonilla, & Leonard, 2017).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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