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Original Article

A pollen fatty acid enhances learning and survival in bumblebees

Felicity Muth,[a] Phillip R. Breslow,[a] Pavel Masek,[b] and Anne S. Leonard[a]

[a]Department of Biology, University of Nevada, Reno, NV 89557 and [b]Department of Biological Sciences, Binghamton University, Binghamton, NY 13902

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Learning associations between food-related stimuli and nutrients allows foragers to collect resources efficiently. In turn, the nutrients that foragers consume can themselves affect learning performance, through innate preferences for pre-ingestive stimuli, as well as post-ingestive reinforcement. Bees are insect models of learning and memory, yet the vast majority of this research concerns nectar (carbohydrate) rather than pollen (protein/lipid) rewards, despite the fact that many bees collect both simultaneously. We asked how one component of pollen surface chemistry, a free fatty acid (oleic acid), affected bees' performance in a nectar-learning task. We found that ingestion of oleic acid enhanced visual learning, likely through positive post-ingestive reinforcement. This was supported by our finding that although bees did not prefer to consume the oleic acid solution, its ingestion both decreased motor activity and increased survival. These results are a step towards understanding how nutritionally complex floral rewards may affect cognitive processes that underlie pollination mutualisms.

Key words: **associative learning, cognition, memory, nutrition, oleic acid, pollinators.**

INTRODUCTION

Generalist foragers often use learning to guide resource selection (Dukas 1998): by associating a particular color, taste, smell, or sound with a nutritional resource, the forager may be more likely to find that food again in the future (Simpson and Raubenheimer 2012). However, learning about a food item can also be affected by properties of the food itself, via processes that play out across multiple timescales. For example, upon encountering the smell or taste of a food item, foods with stimuli of higher motivational value (that taste or smell "better" to the animal) will be learned about more quickly (Shettleworth 2010). After consumption, post-ingestive consequences can also determine the animal's learned response to food-related stimuli (Scott 2011). The most well-studied example of this is conditioned taste aversion, where an animal learns to avoid a food based on its taste (previously neutral or rewarding) after being made to feel ill from a noxious substance (Garcia et al. 1966; Lee and Bernays 1990). Animals can also learn to prefer foods based on positive post-ingestive effects, for example, preferring a particular flavor after it is paired with a tasteless nutrient or nutrient injection (rev. Sclafani 1991). Across longer timescales, chronic nutritional stress can also affect learning and memory performance (Halas et al. 1979; Xia et al. 1997).

Foraging bees provide a model for understanding how both pre- and post-ingestive mechanisms guide learning in an ecological context, because both operate when bees learn associations between floral stimuli and nectar rewards. Nectar with a higher sucrose concentration is innately preferred by bees, and thus conditioned responses to higher concentrations are generally acquired faster and take longer to extinguish (Loo and Bitterman 1992; Cnaani et al. 2006). Once consumed, sugar identity has post-ingestive effects on long-term memory formation separate from its effects on learning (Simcock et al. 2018).

In addition to nectar, bees also collect pollen as their primary source of protein and lipids (Nicolson 2011). As when foraging for nectar, bees contact pollen with chemosensitive mouthparts, antennae, and tarsi while on the flower or during grooming; after packing pollen into corbicular loads, social foragers (*Apis*, *Bombus*) transport it back to the colony where it is critical for larval growth and can contribute to adult survival (Smeets and Duchateau 2003). Floral pollens vary substantially in their nutritional content (Roulston et al. 2000), and foragers of at least some species accordingly select pollens relatively higher in protein or lipids as needed (*Bombus impatiens:* Vaudo et al. 2016).

When foraging for pollen, bumblebees learn floral features associated with its presence (Grüter et al. 2008; Arenas and Farina 2012; Nicholls and Hempel de Ibarra 2014; Muth et al. 2015; Muth et al. 2016b; Russell et al. 2016). Although it is not clear how pollen mediates learned floral associations, both pre- and post-ingestive stimuli may be involved, as is the case for nectar. The "pollenkitt" that coats the surface of many pollen grains (Knoll 1930; Pacini and Hesse 2005) consists of saturated and unsaturated lipids, carotenoids, flavonoids, protein, and carbohydrates (Dobson

Address correspondence to F. Muth. E-mail: fmuth@unr.edu

1988; Hesse 1993; Pacini and Hesse 2005). When foraging, bees are attracted to pollenkitt volatiles (Lepage and Boch 1968; Dobson 1987), and use taste to guide subsequent landing decisions (Muth et al. 2016a). Bees are also able to select pollens based on their protein:lipid ratio (although the mechanisms by which this happens are not clear) (Vaudo et al. 2016). Pollen nutrition also impacts cognitive performance via post-ingestive effects: pollen fatty acids serve as essential dietary components for bees (Manning 2001) and their deficit can impair associative learning performance: Arien et al. (2015) found that honeybees maintained on diets low in omega-3 polyunsaturated fatty acids for 6 weeks performed worse in both olfactory and tactile conditioning assays.

Given that many bees collect both pollen and nectar, it is surprising how little we know about how these 2 rewards together influence learning and memory of floral traits. To date, nearly all research on bee learning has focused exclusively on a single resource, despite the fact that foragers can simultaneously learn about both (Muth et al. 2015; Muth et al. 2017), and given that many bees, like bumblebees, collect both resources at the same time (Free 1955; Francis et al. 2016; Russell et al. 2017). How the smell, taste and/or consumption of pollen affects bees' learning of nectar-based associations is thus unknown, despite the fact that pollen is (nearly) omnipresent when bees learn floral associations (c.f. Table 1 in Muth et al. 2017).

Here, we explore whether and how one of the fatty acids most common to bee-collected pollens, oleic acid (Manning 2001), mediates learning and recall of a visual association. Our design allowed us to identify whether pre-ingestive stimuli associated with this pollenkitt chemical (scent and/or antennal taste) were at play, or whether consumption was required to affect learning (Experiment 1). After finding that consumption of oleic acid, but not its antennal contact, enhanced learning and memory, we sought to determine whether this effect was driven by oral taste (Brito Sanchez et al. 2007) via capillary tube preference assays (Experiment 2). Upon finding no evidence for a taste preference, we explored whether the learning enhancement was due specifically to consumption during learning, or rather a more general physiological effect of the oleic acid ingestion that might have affected learning either directly or indirectly (Experiment 3a). To further explore possible physiological effects, we also addressed oleic acid's effects on bees' activity and survival (Experiment 3b).

GENERAL METHODS

We used 12 colonies (Experiment 1–2, $N = 8$; Experiment 3, $N = 4$) of *Bombus impatiens* (Koppert Biological Systems, Howell, MI), connected to a central foraging arena (L × W × H: 100 × 95 × 90 cm) with ad libitum access to sucrose feeders (15% w/w). Colonies were given 0.6 g of honeybee-collected pollen (Koppert Biological Systems) every other day. We collected foragers from feeders, cold-anesthetized them, and either harnessed them for Proboscis Extension Reflex (PER) conditioning (in Experiment 1, 3a and for survival analysis in Experiment 3b) (using methods similar to Riveros and Gronenberg (2012)) or placed them into individual chambers (in Experiment 2 and for activity monitoring in Experiment 3b).

In all experiments, we presented oleic acid as a 1:200 dilution of oleic acid:sucrose ("FA+S"). The oleic acid was dissolved in ethanol (equal parts) and the sucrose was a 30% (w/w) reagent-grade sucrose solution. All treatments involving a plain 30% sucrose solution ("S") similarly had ethanol added in a 1:200 dilution of ethanol:sucrose.

Data analysis

We analysed all data in R (R Core Team 2018) (details for each experiment below). Where relevant, we initially included interaction terms and then removed them if they were non-significant. To carry out GLMMs we used the lme4 package (Bates et al. 2015) and to carry out LMMs we used the nlme package (Pinheiro et al. 2018). Where we had significant interactions, we used the emmeans package (Lenth 2018) to determine the source of these differences. In cases where our models did not generate *P* values (i.e. for GLMMs), we compared models using the anova() function to carry out a likelihood ratio test between models with and without the variable in question. Further details on statistics used in each experiment are given in each section below.

EXPERIMENT 1: HOW DOES A POLLEN FATTY ACID AFFECT VISUAL LEARNING?

Methods

To test whether oleic acid affected visual learning, we adapted a version of the PER protocol (Bitterman et al. 1983; Riveros and Gronenberg 2009). In this protocol, learning is assessed under conditions that allow for controlled presentation of stimuli while the bee is restrained in a harness. We caught foraging bees and chilled them on ice for 20–25 min before mounting them in plastic tubes (7–8 mm diameter) with a "yoke" to hold their head in place. We left bees to acclimatize to the harness for 2 h (as in Riveros and Gronenberg 2012) at room temperature. We have found that this amount of time is optimal for bees to be sufficiently motivated to partake in PER. After this time, we presented all bees with a small droplet of a 30% sucrose solution via a syringe (presented first to the antennae and then to the proboscis of the bee). Bees that did not exhibit PER were removed from the experiment. Five minutes later, we moved all bees to the training apparatus, located in a dark room under red light.

The PER training apparatus consisted of a circular rotating platform suspended 28 cm above the tabletop (Figure 1). Twelve "training chambers" created from plastic cylinders were affixed to the underside of this platform, approx. 6 cm apart. Each training chamber contained an open "window" (W × H: 3 × 1.5 cm) which allowed the experimenter access to the harnessed bee. Apart from a thin mounting platform, the underside of each training chamber was open, allowing light to project up from a platform below on which 3 blue LED lights (λ = 470 nm) were mounted. Each chamber was lined with foil to disperse light; we controlled whether the lights were on or off via a switchboard.

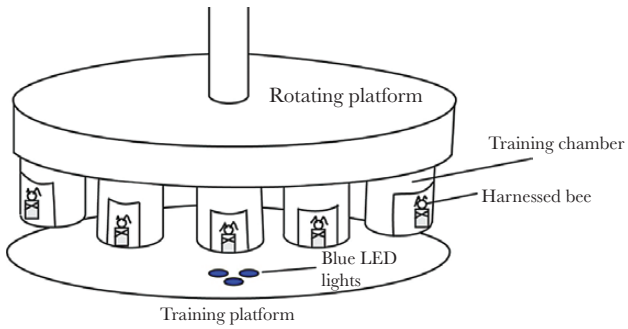


Figure 1
Diagram of the Proboscis Extension Response (PER) training apparatus used in the current study.

During a training trial, each bee was presented with the conditioned stimulus (CS) (a blue light; Figure 2a) followed by 2 unconditioned stimuli, the "pre-ingested reward" and the "ingested reward," offered via a pair of joined syringes (Figure 2b). The 2 reward types were either a sucrose solution alone ("S") or oleic acid in a sucrose solution ("FA+S"), as described in the *General Methods*. The pre-ingested reward in the upper syringe was presented to the bees' antennae, where it could be smelled and tasted with antennal receptors (Haupt 2004). The "ingested reward" in the lower syringe was offered for consumption to the bee's proboscis (Figure 2b) and thus could be potentially assessed both by oral/mouthpart receptors and by post-ingestive feedback.

All bees experienced 8 training trials (inter-trial interval: 5 min), with each trial consisting of the pairing of a blue light CS with the unconditioned stimuli (pre-ingested and ingested rewards). For each reward presentation, we first presented the bee with the pre-ingested reward to its antennae (for ~1 s), and then, if it exhibited PER, we held the ingested reward to its proboscis, allowing it to drink for 3 s (~40 µL ingested over the course of training). After consuming the ingested reward, the blue light was switched off and the reward removed simultaneously (Figure 2c). On trials 2 through 8, we gave bees the rewards as soon as they exhibited PER in response to the blue light (even if this was less than 10 s). In all trials, we recorded whether bees exhibited PER prior to reward presentation and if not, whether they consumed the reward after it was presented to them. 30 min after the eighth trial, we tested bees' memory by recording whether they exhibited a PER after we presented them with the blue light but no reward. We tested a subset of bees at 10 min (and not at 30 min), but did not include these since sample sizes were not sufficient for analysis; resulting sample sizes are shown in Figure 2c.

To determine whether any effects on learning were driven via antennal versus oral contact with the oleic acid, we manipulated the content (S vs. FA +S) of the pre-ingested vs. ingested reward in a factorial manner across 4 experimental treatments (summary and final sample sizes in Figure 2c). To confirm that training produced a conditioned response (i.e. that an increase in PER in response to the CS reflected learning rather simply an increase in the bees' tendency to extend their proboscis over trials), we included 2 backward conditioning treatments as controls. In these treatments, bees were presented with the reward (either S or FA+S; pre-ingested and ingested rewards were the same), followed by the CS, with the same number of trials as the forward-conditioning procedure (final sample sizes in Figure 2c). We tested one block per day and 12 bees in each training block (19 blocks total), resulting in 2 bees per treatment per block.

To determine whether learning differed between the different treatments, we carried out a binomial GLMM with the binary response variable "PER to CS/ no PER to CS" and the explanatory variables "pre-ingested reward" (S or FA+S), "ingested reward" (S or FA+S), trial (1–8) and the random factor "bee." We excluded bees who failed to respond to the rewards more than 2 times (S/S $n = 2$; FA+S/FA+S $n = 8$; S/FA+S $n = 5$; FA+S/S $n = 4$; backward S $n = 2$; backward FA+S $n = 1$). We planned to exclude bees that exhibited PER prior to the presentation of the first reward (i.e. just in response to the blue light) on the first trial, however none did. To determine whether bees differed in their response in the test phase, we carried out a binomial GLM with the response variable "responded/ didn't respond" and the explanatory variables "pre-ingested reward" (S or FA+S) and "ingested reward" (S or FA+S).

Results

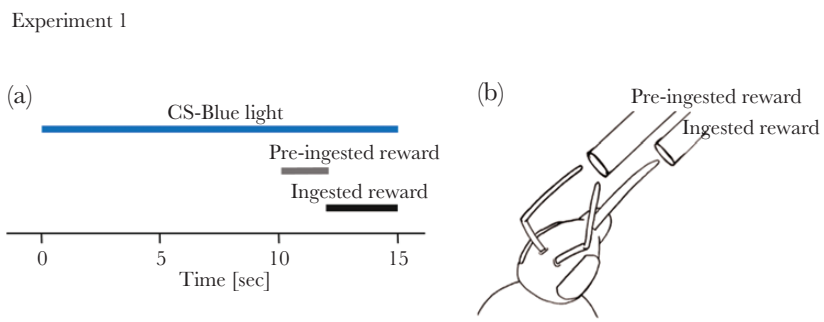
Bees that ingested the FA+S solution during training learned with fewer errors across trials than bees that consumed sucrose only (ingested reward: $z = -2.14$, $P = 0.033$; trial: $z = 10.98$, $P < 0.001$; Figure 4a). Learning was not affected by which reward bees perceived via their antennae (i.e. the pre-ingested reward) ($z = -0.18$; $P = 0.85$; Figure 4a). Bees that consumed the fatty acid solution also trended in the direction of performing better in the memory test (pre-ingested reward: $z = -0.10$; $P = 0.95$; post-ingested reward: $z = -1.89$; $P = 0.058$; Figure 4a).

None of the 59 total subjects in the 2 backward conditioning treatments exhibited PER to the CS, showing that the increased tendency of bees to show PER in our 4 experimental groups reflected a conditioned response rather than an increase in their tendency to extend their proboscis.

EXPERIMENT 2: DO BUMBLEBEES PREFER THE FA+S SOLUTION?

Methods

The observed enhancement to learning in Experiment 1 could either be due to a post-ingestive effect of oleic acid, or because bees preferred the taste (via their mouthparts) of the FA+S solution to the sucrose-only solution, thus making it a more salient reward. To test this second hypothesis, we tested whether individual bees had a preference for FA+S relative to S in a capillary feeding assay (Figure 3). We placed foragers in individual preference chambers (acrylic tubes, OD × L: 25 × 135 mm, TAP Plastics, USA). We



Pre-ingested	Ingested	Training n	Testing n
FA+S	FA+S	33	15
FA+S	S	35	12
S	FA+S	35	14
S	S	38	15

Backward conditioning treatments

S	S	28	6
FA+S	FA+S	28	6

Figure 2
Diagram of (a) training trial timing; (b) administration of pre-ingested and ingested rewards, and (c) sample sizes of treatment groups in Experiment 1. For the backward conditioning treatment groups (controls), bees were presented with rewards prior to the CS.

Figure 3
Diagram of the preference assay. By measuring the distance the meniscus moved from a starting line, we determined how much of 2 solutions the bee consumed over time.

allowed bees to acclimatize to the preference chamber for 24 h during which time they had access to 1.5 mL of 15% sucrose via a wicked feeder. We removed this feeder 2 h before replacing it with 2 capillary tubes (OD × L: 5 × 65mm, World Precision Instruments, USA), spaced 5 mm apart, each filled with 500 µL of a given solution, and plugged with cotton.

This experiment involved 3 treatments: one group chose between the FA+S and S solutions (each 30% sucrose) used in Experiment 1 (n = 22); 2 other groups chose between solutions otherwise identical to FA+S and S, but with 5% (n = 22) or 0% sucrose (water) (n = 23), allowing us to detect possible concentration-dependent responses.

We marked the initial position of each tube's meniscus and measured the distance the meniscus had retracted at 15, 30, 60, 90, 180, and 210 min (1mm ≈ 12µL). We ran this experiment in 4 blocks, with the 3 treatments roughly equally represented in each block. To control for evaporation, we also collected data from otherwise identical but bee-less chambers (one/treatment group/block) and subtracted that (minimal) volume from the other data.

To determine whether bees had a preference for oleic acid in solution, we compared whether bees differed in the amount they consumed of the 2 solutions (for each concentration separately) across time using LMMs with the response variable "amount consumed" and the explanatory variables "solution type" (FA+S or S), "time" (continuous variable) and the random factor "bee."

Results

Bees did not discriminate between tubes filled with S versus FA+S, consuming the same amount of both solutions across time (LMM: solution type: $F_{1, 240}$ = 3.069, P = 0.081; time: $F_{1, 240}$ = 3.069; P < 0.0001; Figure 4b). Similarly, when we tested bees on their preference for 5% sucrose solutions with or without oleic acid, bees did not discriminate (solution type: $F_{1, 240}$ = 0.398, P = 0.850; time: $F_{1, 240}$ = 83.952; P < 0.0001; Supplementary Figure S1). However, when offered a choice between water with or without oleic acid, bees consumed more of the fatty acid solution over time (LMM: solution type*time interaction: $F_{1, 239}$ = 9.290, P = 0.0026; solution type: $F_{1, 239}$ = 15.419, P < 0.0001; time: $F_{1, 239}$ = 85.287, P < 0.0001; Supplementary Figure S2). By changing "time" to a factor and carrying out a post hoc test (in emmeans), we determined that the differences between treatments were apparent at 180 and 210 min.

EXPERIMENT 3A: DOES CONSUMPTION OF OLEIC ACID PRIOR TO LEARNING ENHANCE PERFORMANCE?

Methods

In Experiment 1, we found that consumption of oleic acid during training enhanced learning; and in Experiment 2, we found that this was not due to the bees preferring its taste in sucrose of the concentration we used. We then aimed to determine whether the enhancement to learning found in Experiment 1 was due specifically to the oleic acid being consumed during conditioning (i.e. through post-ingestive reinforcement) or rather whether the enhancement to learning we saw might have been due to a more general physiological effect of the oleic acid on the bee. If the latter, we expected to observe an analogous enhancement when bees were fed the oleic acid solution shortly before (but, crucially, not "during") training.

We harnessed bees for PER conditioning as in Experiment 1, this time pre-fed them either 50 µL S (N = 26) or 50 µL FA+S (N = 26). After 15 min, we trained these bees using the same number of trials and reward timing as Experiment 1 (Figure 2a); in this iteration, we presented bees with a single unconditioned stimulus, 30% sucrose solution, offered via a single syringe. As in Experiment 1, we tested a subset of bees 30 min after training for memory retention (S n = 14; FA+S n = 13) and carried out 2 backward conditioning control treatments (where bees were rewarded before being presented with the CS; S: N = 6; FA+S: N = 8).

To determine whether learning differed between bees that had consumed FA+S versus S prior to training, we carried out a binomial GLMM with the binary response variable "PER to CS/ no PER to CS" and the explanatory variables "pre-fed reward" (S or FA+S), trial (1–8) and the random factor "bee." We excluded bees who failed to respond to the rewards more than twice (S n = 9; FA+S n = 9; backward control S n = 0; backward control FA+S n = 0). To determine whether the test phase response differed we carried out a binomial GLM with the response "responded/ didn't respond" and the explanatory variable "pre-fed reward" (S or FA+S).

Results

Bees that consumed the FA+S solution 15 min prior to training performed the same as bees that were pre-fed S alone (pre-fed reward: z =−0.973, P = 0.33; trial: z = 6.24 P < 0.0001; Figure 4c), and there was no difference in test performance (z =−0.570; P = 0.57; Figure 4c). As in Experiment 1, none of the 14 subjects assigned to the 2 backward conditioning treatments exhibited PER to the CS, ruling out the possibility that oleic acid consumption altered bees' tendency to show the PER by simply increasing responsiveness.

EXPERIMENT 3B: DOES CONSUMPTION OF OLEIC ACID HAVE POST-INGESTIVE EFFECTS ON ACTIVITY AND SURVIVAL?

Methods

After finding that consumption of fatty acid during conditioning enhanced learning (Exp 1), probably through positive post-ingestive reinforcement (Expts 2 and 3a), we then aimed to determine whether there might be any additional physiological effects indicative of post-ingestive effects. Specifically we asked whether consumption of oleic acid at the dose used in learning experiments altered 2 additional metrics where we might expect such physiological effects to manifest: motor activity and/or survival.

Activity

To measure effects on motor activity, we placed 38 foragers in square-ended tubes (L × W × H: 127 × 26 × 26 mm), and left them to sit for 2 h on a white sheet of paper with a line marked in black, marking the middle of the tube. Each bee was then fed 50 µL of one of the 2 solutions as in Experiment 3a, S (N = 17) or FA+S (N = 21). After 15 min, we filmed bees for 1 h and later scored these videos to compare the number of times an individual

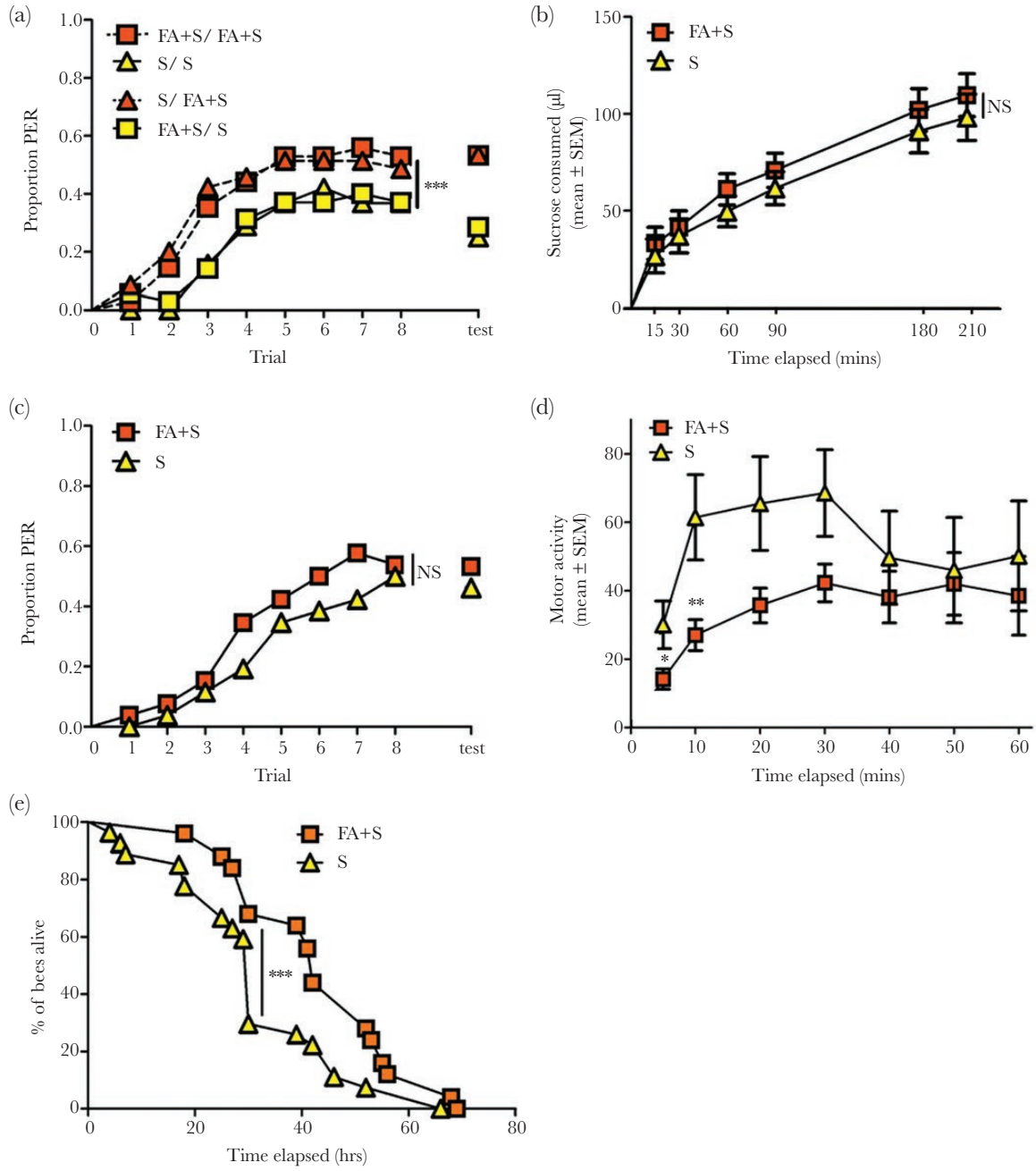


Figure 4

(a) Experiment 1: The effect of oleic acid on learning performance, measured as the proportion of bees exhibiting PER in the 4 treatment groups in response to a conditioned light stimulus, either when trained with sucrose ("S") or oleic acid in sucrose ("FA+S") as rewards. The "pre-ingested reward" (presented to antennae) is listed first (triangle=S; square= FA+S), and the "ingested reward" (presented to proboscis) second (yellow = S; orange = FA+S). (b) Experiment 2: The preference of bees for FA+S versus S (30% concentration), measured as the cumulative amount of each solution consumed by bees across time when offered a simultaneous choice. (c) Experiment 3a: The effect of previously ingested oleic acid on learning, measured as the proportion of bees exhibiting PER in response to a conditioned light stimulus in a visual learning association task when they had previously been fed either FA+S or S. (d) Experiment 3b: The activity (measured as the number of times bees crossed a center line) of bees fed either FA+S or S across a 60-min period; data shown are non-cumulative counts within each 10-min block period. (e) Experiment 3b: Survival curves for bees that were fed S or FA+S. Statistical significance denoted: *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$.

bee crossed the centre line, as an activity index (a common measure of activity in bees and other insects; Pfeiffenberger et al. 2010).

We carried out a GLMM with a Poisson distribution with the response variable "activity" (number of times the bee crosses the black line) and the explanatory variables: "treatment" (S or FA+S), time block (0−10 min, 10–20 min, 20–30 min, 30–40 min, 40–50 min, 50–60 min), and their interaction term. We also included "bee" as a random factor, to control for the multiple measures per bee across time.

Survival

To determine whether fatty acid consumption affected survival, we harnessed 4–12 bees in each block following the same procedure as

Experiment 1 to replicate any physiological effects (e.g. stress) associated with this protocol (7 blocks total; $n = 52$). After acclimatizing for 3 h, we fed each bee 80 µL of either 30% S or FA+S via a Hamilton syringe (all bees consumed their dose). We then checked the bees hourly for the next 72 h and recorded survival time (bees that died overnight were recorded as having died at the last hour they were checked).

We carried out a GLM with the response variable "number of hours until death" with the explanatory factor "treatment" (S or FA+S). We also carried out a Kaplan-Meier survival analysis to compare the 2 treatments (not accounting for block) which showed the same results.

Results

Activity

Bees that were fed oleic acid in sucrose solution were less active than the control group at the start of the observation period (25 min after initially being fed the FA+S), although across the hour-long observation period the 2 groups converged in their activity levels (Figure 4d: GLMM: significant treatment×time block interaction; comparison of models with and without interaction term: $\chi^2_5 = 144.08$; $P < 0.0001$). Post hoc comparison revealed significant differences between treatments at the first time point (0–10 min): emmean ±SE: FA+S: 3.06 ± 0.18; S: 3.73 ± 0.20). To determine whether these differences were evident at an earlier time point, we also compared the groups at 5 min (the first time point at which we had sufficient data to compare between treatments), and found that they did differ (*t*-test: $t = 2.25$; $P = 0.031$).

Survival

Bees that consumed the fatty acid solution survived for significantly longer than bees that consumed sucrose only (mean h ± SD: FA+S: 43.8 ± 14.5; S: 31.3 ± 15.6; GLM: $F_{1, 44} = 15.070$, $P < 0.001$). A Kaplan-Meier survival analysis also confirmed that bees that consumed the fatty acid were more likely to survive for longer ($\chi^2_1 = 6.947$, $P < 0.01$; Figure 4e).

DISCUSSION

Although the vast majority of previous research on bee cognition focuses on nectar foraging, most species of bee collect both nectar and pollen, and within *Bombus* species, individuals often collect nectar and pollen on a foraging bout and from a single flower (Muth et al. 2015; Francis et al. 2016; Muth et al. 2017). While there has been recent interest in understanding how bees detect (Dobson and Bergström 2000; Lunau 2000), select (Vaudo et al. 2016), and learn about pollen rewards (Nicholls and Hempel de Ibarra 2014; Muth et al. 2015; Muth et al. 2016b), this work usually considers learning in a single reward context. Thus, most of the basic sensory and cognitive aspects of how bees forage for pollen in conjunction with nectar remain obscure (Leonard and Francis 2017). Understanding how bees forage in light of the nutritional complexity of rewards is particularly pertinent given that nutritional stress and loss of pollen resources are a major contributor to bee declines (Goulson et al. 2005; Goulson et al. 2015; Vaudo et al. 2015).

Here, we investigated one facet of the nutritional complexity faced by foraging bees: we asked how a common pollen surface chemical affected learning visual associations. We found that when oleic acid was used as a reward (in sucrose solution) during training, learning was enhanced. This enhancement did not seem to be driven by pre-ingestive cues (i.e. scent or taste), since antennal stimulation with oleic acid did not enhance learning, and since bees did not prefer the fatty acid solution over the control solution. The lack of a demonstrated preference for the FA+S solution could be either due to bees valuing it equally to the control solution, or due to an inability to detect the oleic acid in solution. Despite clear physiological effects of the fatty acid (on activity and survival), the learning enhancement did not seem to be due to a direct effect of the fatty acid on the ability to learn, since ingesting the fatty acid prior to learning did not enhance performance. Taken together, these findings suggest that the fatty acid had a positive post-ingestive effect, increasing the value of the reward to the bee: bees were more motivated to learn associations with it, and thus learning performance was improved. Reinforcement from positive post-ingestive nutritional consequences has been found in other cases: rats fed flavored water followed by a nutrient (such as starch) develop a conditioned preference for that flavor (Capaldi et al. 1987; Elizalde and Sclafani 1988). Similarly, rats fed a flavor followed by a nutrient via intragastric infusions (to avoid any potential confounding effects of the nutrient taste), also develop conditioned preferences for the flavor (Baker et al. 1987; Elizalde and Sclafani 1990; reviewed in Sclafani 1991). Evidence for positive post-ingestive effects have also been found in invertebrates, where both palatability and nutrient value of sugars contribute to the reinforcement of appetitive memory (*Drosophila*: Burke and Waddell 2011; *Apis mellifera*: Simcock et al. 2018). Our study indicates that other nutrients beyond sugars may also reinforce learning in bees through post-ingestive reinforcement.

If our results are indeed due to bees pairing the conditioned visual cue with the nutritional consequences of a fatty acid (in sucrose) reward, then we would expect that the post-ingestive nutrient detection takes place in less than 10 min (the time at which we detect differences in Experiment 1). The timeline of lipid metabolism has not (to our knowledge) been determined for bees (rev. Turunen and Crailsheim 1996; Canavoso et al. 2001), but research in other insect systems suggests that absorption, transport to hemolymph, and metabolic turnover can occur on timescales relevant to our behavioral experiments (Tsuchida and Wells 1988; Soulages and Wells 1994; Atella et al. 2000; Arrese et al. 2001). At least in terms of activity, we found effects at 20 min post consumption, if not before (we only started measuring activity 15 min after initial consumption and it took 5 min from this point to have sufficient data to compare treatments).

We cannot rule out the possibility that the enhancement to learning that we found in Experiment 1 was also due to a pre-ingestive cue, likely taste. This would have to be oral, rather than antennal taste, given that bees stimulated with oleic acid on the antennae did not show any enhancement relative to bees that were stimulated with sucrose alone. It would also have to be a preference only expressed in the harnessed PER procedure, and not observable in our free-moving preference assay. This explanation seems unlikely, because although previous studies have found that harnessed bees do have different taste preferences than free-moving bees, they are generally less responsive to taste stimuli: they accept higher concentrations of sucrose relative to free-moving bees (Mujagic and Erber 2009) and are more likely to ingest toxic substances (Ayestaran et al. 2010). It is also possible that our preference assay was not sensitive enough to detect taste preferences. However, this too seems unlikely, since we did detect preferences between water and water containing oleic acid (similar to findings from *Drosophila melanogaster* (Masek and Keene 2013)). Instead the presence of sucrose seemed to mask any taste preferences for fatty acid. Finally, one might expect that

positive post-ingestive feedback from the oleic acid solution would have generated a preference in our assay, even if bees cannot taste it. However, given that in this assay bees sample 2 solutions in close proximity, it is unlikely that bees are able to determine which solution is responsible for any post-ingestive effects.

Despite not finding that consumption of oleic acid prior to learning altered learning performance, we did see clear effects of the oleic acid on 2 other measures: activity and survival. Oleic acid may have affected bees' activity through providing additional nutrients, with the bees' lowered activity being evidence of satiation. Additional beneficial nutrition may have promoted the longer-term survival we found (as it is known to do in other invertebrate systems: *Drosophila melanogaster* (Masek and Keene 2013) and *C. elegans* (Han et al. 2017). It is also possible that the reduction in activity reduced energy expenditure, and thus resulted in bees surviving longer. However, these physiological effects do not seem to have directly affected learning, otherwise we would have expected to see an enhancement to learning in the bees fed oleic acid prior to conditioning. Similarly, if oleic acid had enhanced learning via direct effects on the nervous or endocrine systems (i.e. honeybees fed a diet low in omega-3 polyunsaturated fatty acids perform worse in learning assays (Arien et al. 2015)), we would have expected a similar effect in bees fed oleic acid prior to conditioning.

Regardless of the underlying mechanism/s, our results suggest that pollen foraging might affect colony fitness not only through its well-established direct effects on growth, survival, and reproduction (Schmidt et al. 1987; Genissel and Aupinel 2002; Di Pasquale et al. 2013), but also indirectly through affecting bees' tendency to learn associations between floral stimuli and rewards. Promising next steps would be to investigate whether other features of pollen chemistry similarly promote learning of floral features and to explore the possibility for synergistic effects. Although the PER protocol we used in this study is useful for tightly controlling the order of stimulus and reward presentation and is thus an advantageous tool for testing effects on learning under controlled conditions, in pilot work, we found that fatty acids needed to be dissolved in sucrose to elicit PER in bumblebee foragers. This is in contrast to previous work with honeybees, that found that various constituents of pollen (including fatty acids), elicited PER in pollen-foraging honeybees and could act as unconditioned stimuli in the absence of nectar (Arenas and Farina 2012). In order to explore how nectar and pollen interact to affect floral learning in a more natural foraging scenario, bees could be tested in scenarios involving artificial flowers containing nectar and pollen surrogates of controlled composition (by adding chemicals of interest to standardized pollen as in Muth et al. 2016a) or pollen substitutes (e.g. α-cellulose). While it is clear that bees do learn about pollen while foraging (Grüter et al. 2008; Arenas and Farina 2012; Nicholls and Hempel de Ibarra 2013; Nicholls and Hempel de Ibarra 2014; Muth et al. 2015; Muth et al. 2016b; Russell et al. 2016), our findings raise the question of what timescales pollen might affect learning. While bees can taste pollen (Ruedenauer et al. 2015; Muth et al. 2016a), they can also discriminate between pollens based on nutrient ratios (Vaudo et al. 2016). The mechanism driving this discrimination among pollens of different lipid composition is currently unclear, but our work suggests that post-ingestive feedback could play a role. Future work might address whether the positive post-ingestive effects we found evidence for in this study play a role in foragers' assessing the nutritional quality of pollen.

The ability to learn associations with floral features can be an important contributor to foraging efficiency and, ultimately, colony-level performance (Raine and Chittka 2008). However, it is often studied in fairly artificial environments using sucrose as a nectar surrogate, ignoring both the effects of secondary nectar compounds (Simcock et al. 2014; Richardson et al. 2015) and the role of pollen (Muth et al. 2017). The little we do know about how these 2 rewards interact to affect pollinator learning suggest a host of intriguing interactions: for example, nectar can impair learning of pollen-color associations (Muth et al. 2017), and visual stimuli associated with pollen can impair learning of nectar-color associations (Pohl et al. 2008). Integrating aspects of ecologically relevant learning scenarios will not only yield insights into how floral rewards interact to govern bee learning and memory, but will also expand bees' role as a more general model for understanding complex linkages between nutrition and cognition.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online

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Data accessibility: Analyses reported in this article can be reproduced using the data provided by Muth et al. (2018).

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