



Appetitive olfactory learning and memory in the honeybee depend on sugar reward identity



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ABSTRACT

One of the most important tasks of the brain is to learn and remember information associated with food. Studies in mice and *Drosophila* have shown that sugar rewards must be metabolisable to form lasting memories, but few other animals have been studied. Here, we trained adult, worker honeybees (*Apis mellifera*) in two olfactory tasks (massed and spaced conditioning) known to affect memory formation to test how the schedule of reinforcement and the nature of a sugar reward affected learning and memory. The antennae and mouthparts of honeybees were most sensitive to sucrose but glucose and fructose were equally phagostimulatory. Whether or not bees could learn the tasks depended on sugar identity and concentration. However, only bees rewarded with glucose or sucrose formed robust long-term memory. This was true for bees trained in both the massed and spaced conditioning tasks. Honeybees fed with glucose or fructose exhibited a surge in haemolymph sugar of greater than 120 mM within 30 s that remained elevated for as long as 20 min after a single feeding event. For bees fed with sucrose, this change in haemolymph glucose and fructose occurred with a 30 s delay. Our data showed that olfactory learning in honeybees was affected by sugar identity and concentration, but that olfactory memory was most strongly affected by sugar identity. Taken together, these data suggest that the neural mechanisms involved in memory formation sense rapid changes in haemolymph glucose that occur during and after conditioning.

1. Introduction

The brain has been shaped by natural selection to learn to associate cues that predict the occurrence of nutritiously valuable food. Sensory input is organized to produce memory traces for food that are stored for retrieval when animals are hungry, so that animals can identify signals associated with nutritional rewards and avoid signals that are irrelevant or that are associated with intoxication. An important mechanism for assessing food value and forming lasting memories of sensory cues is through post-ingestive signalling. This was first studied in the context of aversion learning; within one trial, animals can learn to associate tastes and smells with the post-ingestive consequences of ingesting toxins in foods (Bernays and Lee, 1988; Garcia et al., 1955; Wright et al., 2010). More recently, experiments with mice and fruit flies have shown that post-ingestive signals are important for assessing the nutritional value of food; memories last longer when foods have metabolic value (Burke and Waddell, 2011; de Araujo et al., 2008; Dus et al., 2011; Fujita and Tanimura, 2011; Sclafani and Ackroff, 2016). For example, insects trained in an olfactory learning task with a non-metabolisable sugar such as arabinose can learn to associate an odour with the taste of this sugar, but they do not form long-lasting memories of the odour (Burke

and Waddell, 2011).

Memories of food should reflect food value: learning should happen faster and memories should be stronger and longer lasting for high valence rewards (Pavlov, 1927). Few studies have tested how reward quality affects learning and memory, and whether all metabolisable sugars are equally rewarding to animals. Mice are more likely to learn and remember when they are rewarded with sugars metabolised into glucose-units but not when rewarded with fructose (Matsumura et al., 2010; Sclafani and Ackroff, 2016). In contrast, studies in *Drosophila* indicate that flies form lasting memories for several metabolisable sugars including fructose and glucose (Burke and Waddell, 2011; Dus et al., 2013, 2011; Miyamoto et al., 2012; Musso et al., 2015; Perisse et al., 2013). This could indicate that the mechanisms of post-ingestive nutrient detection or memory formation in insects and mammals are different.

The honeybee learns to associate floral signals with reward very quickly, and is an important insect model for studying learning and memory (Bitterman et al., 1983; Eisenhardt, 2014; Stollhoff et al., 2008). Our previous work indicated that like *Drosophila*, honeybees also require a metabolic reward to form a lasting olfactory memory of odours associated with food (Wright et al., 2007). Specifically, we

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found that the taste of the reward was not sufficient for long-term memory: only honeybees that had been fed with a sucrose reward exhibited memories that lasted longer than 10 min. This work implied that to form olfactory memories, the bee brain also requires a post-ingestive, metabolic reward, but this has not been explicitly shown.

Previous work in honeybees also showed that time interval between conditioning trials also affects the formation of long-term memory (Menzel et al., 2001). When bees are trained in a ‘massed’ conditioning task (i.e. inter-trial interval of 30 s) and rewarded with sucrose, they are less likely to remember the conditioned odour than bees trained in a ‘spaced’ conditioning task (i.e. inter-trial interval of 3–10 min). Furthermore, single conditioning trial where an odour stimulus lasts ~4 s and is paired with an equally brief, but metabolisable food reward does not produce a lasting memory in bees (Stollhoff et al., 2008). Instead, several trials with inter-trial intervals of > 1 min are necessary (Menzel et al., 2001; Stollhoff et al., 2008). This suggests that the neurons encoding long-term olfactory memory must receive sensory input on a time scale that overlaps or occurs soon after a period of flux in haemolymph nutrients. The fact that bees form lasting memories when they receive several conditioning trials with long inter-trial intervals could indicate that memory formation depends on the timing of post-ingestive reinforcement relative to sensory input but this has not yet been tested in any animal.

Here, we tested whether long-term olfactory memory in honeybees depends upon the nature of the metabolisable sugar, its value/concentration, and the inter-trial interval. Bees were conditioned to associate an odour stimulus with a food reward in a spaced (5 min inter-trial interval) or massed (30 s inter-trial interval) task for conditioned proboscis extension response (PER). After training, all bees were tested for their short-term (10 min) and long-term (24 h) olfactory memory with the conditioned odour and a novel odour (NO). With the aim of identifying how haemolymph sugar flux could influence learning and memory, we also measured the amount of time necessary for post-ingestive changes in haemolymph sugars to occur.

2. Methods

Animals: Worker honey bees (*Apis mellifera* var *carnica* or *Apis mellifera* var *buckfast*) were captured during April–August 2011 and 2012 from a hive located at Newcastle University (UK) as they returned from foraging. A plastic blockade was placed over the hive entrance to ensure only returning foragers were captured. Each bee was collected in a plastic vial and restrained in a harness as described in Wright et al. (2007). Bees were used either for the gustatory assays, haemolymph collection or for olfactory conditioning; each bee was fed to satiety with 1.0 M sucrose and left for 18–24 h at room temperature (RT) in a humidified plastic box.

2.1. Gustatory assays

Bees from this experiment were captured during April–May 2011. **Antennal assay:** The antennae of each honeybee was stimulated with an ascending concentration series (0.3, 0.6, 1.0, 1.3, 1.6 and 2.0 M) of sucrose, fructose, or glucose to elicit the PER. Between each stimulation, each bee was tested for its response to water as described in (Page et al., 1998). Stimuli were applied such that an interval of 3–5 min occurred between each stimulus to avoid producing habituation to the test stimuli. All bees were tested with each series of each sugar. A total of 140 bees were tested; 50 of them did not respond to any of the stimuli. **Mouthparts assay:** Each bee was tested with a water stimulus and one concentration of each sugar as using the assay for proboscis sensitivity previously described in Wright et al. (2010). We tested individual bees with one concentration of each sugar; this was done to avoid alterations to motivation state that could confound the experiments when the bees ate the solutions. (Note: motivation state to respond to the solution is not altered in bees who have had their antennae

touched with the solution only, as in the antennal assay). To accomplish the application of the solution to the mouthparts, the antennae were first stimulated with the test solution to elicit proboscis extension. The test stimulus was then applied to the mouthparts. Whether or not the bee consumed the solution was recorded as a binary variable. Bees that did not respond to antennal stimulation were not used in the experiment. Between 0 and 50% of the subjects did not respond during this assay, depending on the stimulus used as the test stimulus (total N/treatment = 20, only data for bees that responded to antennal stimulation is plotted).

2.2. Olfactory conditioning

Bees from this experiment were captured during June–August 2012. After 24 h, each bee was trained in a protocol for olfactory conditioning of the PER (Bitterman et al., 1983). Methods for odour stimulus delivery are described in Wright et al. (2007). Only subjects that responded with PER to antennal stimulation with 1.0 M sucrose were selected for conditioning. Bees were conditioned for 6 trials with an inter-trial interval (ITI) of 30 s (massed conditioning) or 5 min (spaced conditioning). The conditioned stimulus (CS) was 1-hexanol (Sigma-Aldrich) and was presented for 4 s. The unconditioned stimulus (food reward, US) presented on each trial was a 0.4 µl droplet of reagent-grade fructose, glucose or sucrose delivered using a Gilmont syringe (Cole Parmer). We also tested 3 concentrations of each sugar: 0.3 M, 1.0 M, and 2.0 M. Any bee that responded with a conditioned response on the first trial was removed from the experiment during the experiment. Two unreinforced olfactory memory tests were administered 10 min and 24 h after olfactory conditioning: one with the CS odour and one with a novel odour (2-octanone, Sigma-Aldrich). The order of presentation of the test odours was randomized across subjects. Each treatment group was randomized across the course of the study; on any given day, at least 3 treatment groups were trained and tested.

2.3. Haemolymph analysis

Honeybees were individually harnessed as described above and a small incision was made above the median ocellus using a 1.1 mm × 40 mm needle (BD Microlance). Honeybees were split into one of four experimental groups and fed: 5 µl of 1.0 M sucrose, 1.0 M glucose, or 1.0 M fructose or fed to satiety with 1.0 M sucrose. (Note: for the bees fed to satiety, the time taken for each bee to feed to satiety was recorded in order to gauge the change in sugar levels from the initiation and termination of feeding). At a specific time point post-feeding, haemolymph was collected using a 10 µl capillary tube (Hirschmann) from the incision above the median ocellus. The haemolymph was sampled at one of the following time points: 30 s, 1 min, 3 min, 5 min, 10 min and 20 min post-feeding. Each capillary tube was placed in the head capsule for a total of 2 min after the specified time point. Haemolymph was also collected from a subset of bees prior to feeding (time point zero). A minimum of 1 µl of haemolymph was collected for each bee and immediately added to 1 µl 0.1 M perchloric acid; any volume greater than 1 µl was matched with an equal volume of 0.1 M perchloric acid and subsequently stored at –20 °C until further processing. Samples less than 1 µl were discarded, as was any haemolymph available after the 2 min collection time in order to standardise all samples. Haemolymph samples were taken from 10 bees per treatment group and analysed using HPLC.

Haemolymph samples were centrifuged for 10 min at 14,000 rpm (Eppendorf model no. 5424), and 1 µl of the haemolymph supernatant was removed and diluted 1:200 with nanopure water (Fisher Scientific). Diluted samples were filtered through a syringe filter (Puradisc sample preparation nylon 0.45 µm pore, 4 mm diameter, Whatman). High performance liquid chromatography (HPLC) was used to measure concentrations of specific sugars (glucose, fructose, sucrose and trehalose) in each sample. HPLC analysis was conducted by injecting 20 µl of

diluted sample via a Rheodyne valve onto a Carbowax PA-100 column (Dionex, Sunnyvale, California, USA). Sample components were eluted from the column isocratically using 100 mM NaOH flowing at 1 ml/min. The chromatographic profile was recorded using pulsed amperometric detection (ED40 electrochemical detector, Dionex). Elution profiles were analysed using PeakNET software package (Dionex, Breda, The Netherlands). Daily reference curves were obtained for glucose, fructose, sucrose and trehalose by injecting calibration standards with concentrations of 10 ppm for each sugar.

2.4. Statistics

All data were analysed using SPSS v 23. All modelling was done such that only significant factors in the analysis are reported; when higher order terms in models were not significant, they were removed from the model in a stepwise manner. Taste assays: Data for the antennal assay were analysed using generalized estimating equations (GEE) for repeated-measures logistic regression because each bee was stimulated multiple times. Bees that did not respond to any trial were not included in the analysis (50 out of 140 subjects). Data for the mouthparts assay was analysed as a generalized linear model (GLM) using binary logistic regression because each bee was tested with one solution only and could be treated as an independent case. For the taste assays, the independent variables were sugar type and concentration; the response variable was scored as a binary variable. Conditioning and memory assays: The number of bees that responded with a conditioned response (CR) on at least one trial were defined as ‘responders’ or ‘non-responders’ (a binary variable) and also analysed via logistic regression in GLM. Bees that did not exhibit a learned response (i.e. did not respond with a conditioned PER on any of the trials) were excluded from the analysis of the rate of learning and the memory recall test. This filter was applied to permit an accurate assessment of the performance of the honeybees that learned, because the inclusion of ‘non-responding’ bees biases the memory data and does not accurately reflect ‘memory’ – i.e. that bees learned the task and did not remember vs. bees that simply did not learn or respond (see Williamson and Wright, 2013; Wright et al., 2015)). The conditioning data was analysed as a sum of responses over 6 trials using a linear model in GLM. The test data were analysed as a binary response variable using logistic regression in GLM. For the test data, the CS-only data were analysed separately for the massed and spaced tasks for ease of interpretation; three-way models including sugar, concentration, and time of the test were originally fitted to the data, with non-significant higher order interactions removed in a stepwise manner. All *post hoc* comparisons were performed using a least-squares pairwise comparison (LSD). Data for the proportions of the responses during the test with the CS and NO were analysed using a Wilcoxon’s signed-rank test. Haemolymph data were analysed using a 2-way MANOVA.

3. Results

3.1. Sucrose is a stronger phagostimulant than fructose or glucose

We tested the antennae and mouthparts of individual honeybees for their response to a concentration series of sucrose, glucose and fructose to identify the phagostimulatory strength of each type of sugar. The antennae were assessed by applying an ascending concentration series of a stimulus to the antennae and measuring whether it evoked the PER. In the antennal assay, the stimulus-response function depended both on the sugar used as the stimulus and its concentration (Fig. 1A, Table S1, conc \times sugar: $\chi^2_{9^2} = 4141$, $P < 0.001$). The antennae were most sensitive to sucrose, then glucose, then fructose (Fig. 1A, LSD, all $P < 0.05$). The stimulus-response function to the water control stimulus was the same over trials for all three sugars tested (data not shown, Table S1, conc \times sugar: $\chi^2_{8^2} = 8.38$, $P = 0.397$), though the average value of the response was greatest for bees tested with fructose,

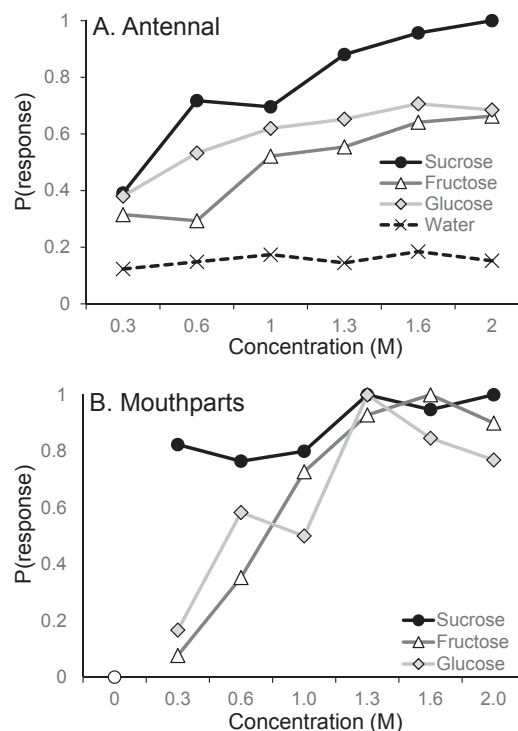


Fig. 1. Gustatory sensitivity assays for the (A) antennae or (B) the mouthparts of adult worker honeybees. A. Honeybees were most likely to extend the proboscis when stimulated on the antennae with sucrose, then glucose, then fructose (LSD, all pairwise comparisons, $P < 0.05$). Note: the average response to the water stimulus over all the trials is represented; the x-axis for the water response corresponds to the test with water that occurred prior to the test with the stimulus at each concentration. All subjects were tested with all stimuli, $N = 90$. B. Honeybees were more likely to consume droplets of sucrose solution than fructose (LSD, $P = 0.018$) or glucose (LSD, $P = 0.002$). Most bees consumed droplets of solution at concentrations greater than 1.3 M of all three sugars. All bees were tested with water (0 M) prior to testing with a sugar stimulus. Sample size for each sugar for each concentration: N_{sucrose} : 0.3 M = 17, 0.6 M = 17, 1.0 M = 20, 1.3 M = 19, 1.6 M = 19, 2.0 M = 20; N_{fructose} : 0.3 M = 12, 0.6 M = 17, 1.0 M = 11, 1.3 M = 14, 1.6 M = 13, 2.0 M = 20; N_{glucose} : 0.3 M = 12, 0.6 M = 12, 1.0 M = 12, 1.3 M = 10, 1.6 M = 13, 2.0 M = 13.

then glucose, then sucrose (Table S1, sugar: $\chi^2_{2^2} = 7.51$, $P = 0.023$, all LSD, $P < 0.05$).

The mouthparts of bees were much more sensitive to sucrose than to fructose or glucose (Fig. 1B), with as many as 80% being willing to consume a droplet of 0.3 M sucrose whereas $< 20\%$ of the bees would consume a 0.3 M droplet of glucose or fructose (Fig. 1B, Table S2, sugar: $\chi^2_{5^2} = 38.2$, $P < 0.001$). Bees were more likely to consume each solution when the concentration increased (conc: $\chi^2_{2^2} = 11.8$, $P = 0.003$); at a concentration of ≥ 1.3 M, all the solutions were consumed (Fig. 1B). None of the bees consumed the droplet of water.

3.2. Bees trained with glucose and sucrose are more apt to learn and learn faster

We first examined the proportion of subjects that could perform the massed or spaced learning task (Fig. 2). The number of honeybees that could acquire the CS-US association depended on the task, the sugar used as the US, and the US concentration (Fig. 2, Table S3, GLM: task \times conc \times sugar: $\chi^2_{4^2} = 12.8$, $P = 0.012$, Table 1). In general, honeybees were more likely to learn to associate an odour with reward when it was reinforced with glucose or sucrose (*post hoc* gluc vs fruct: $P < 0.001$, suc vs fruct: $P < 0.001$, gluc vs suc: $P = 0.269$) and when the US concentration was 1.0 M or greater (*post hoc*: 0.3 M vs 1 M: $P < 0.001$; 0.3 M vs 2 M: $P < 0.001$, 1 M vs 2 M: $P = 0.451$). Most ($\sim 70\%$) of the bees trained with sucrose were able to perform the massed learning task regardless of US concentration. In contrast, less

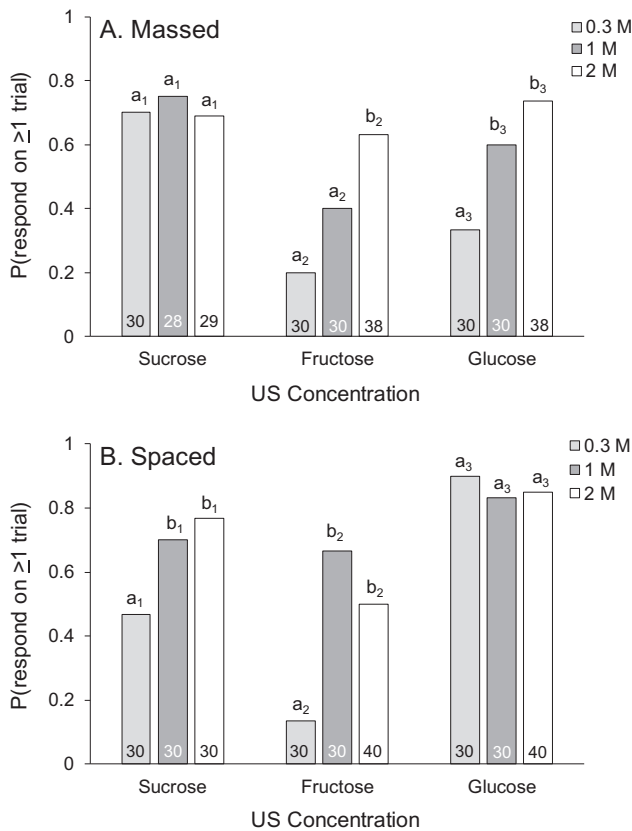


Fig. 2. Proportion of honeybees that learned on at least 1 trial during a 6-trial, massed (A) or spaced (B) olfactory conditioning task. Most bees trained with sucrose solution learned the massed conditioning task, whereas most learned the spaced conditioning task if rewarded with glucose. Massed-conditioned bees rewarded with low concentrations of fructose or glucose were less likely to learn; reward concentration also affected performance of the spaced-conditioned bees rewarded with fructose or sucrose. Letter differences indicate a pairwise-comparison with $P < 0.05$; subscripts refer to comparisons made within a specific treatment. Numbers on each bar indicate sample size per group.

Table 1
Generalized linear model for performance during the conditioning task in Fig. 2.

	Wald χ^2	df	P-value
(Intercept)	22.4	1	< 0.001
sugar	40.6	2	< 0.001
conc	19.7	2	< 0.001
task	5.1	1	0.025
sugar * conc	7.7	4	0.102
sugar * task	16.0	2	< 0.001
conc * task	1.2	2	0.553
sugar * conc * task	12.8	4	0.012

than 50% of the bees trained with low concentrations of fructose (0.3 M or 1.0 M) or glucose (0.3 M) acquired the massed learning task (Fig. 2A). More of the honeybees were able to acquire the spaced learning task; at least 50% of the bees trained with 1.0 M or greater concentrations of any of the sugars learned (Fig. 2B). Surprisingly, most of the bees trained in the spaced learning task with glucose learned (> 80%, Fig. 2B), regardless of its concentration. In both tasks, bees trained with a fructose US were the least likely to learn.

We separated out the bees in Fig. 2 that learned on at least 1 trial and examined the rate of acquisition over the 6 conditioning trials; the speed of acquisition depended on the task and the US concentration (Fig. S1, Table S4, GLM: task \times conc: $\chi^2_2 = 8.82$, $P = 0.012$).

3.3. Bees remember learned odours when fed with glucose but not with fructose

The nature of the reward affected how the bees performed during the short-term memory (10 min) and long-term memory (24 h) tests (Fig. 3). Because of the difficulty in interpreting a 4-way model, we analysed the test data for massed and spaced conditioning separately. In general, honeybees were more likely to respond to the CS odour when the concentration of the US was above 1 M (Table S5, GLM: massed, conc: $\chi^2_2 = 19.9$, $P < 0.001$; spaced, conc: $\chi^2_2 = 11.9$, $P = 0.003$), the US was sucrose or glucose (Table S5, GLM: massed, sugar: $\chi^2_2 = 24.6$, $P < 0.001$; spaced, sugar: $\chi^2_2 = 24.9$, $P < 0.001$), and the test time was 10 min (Table S5, GLM: massed, when: $\chi^2_2 = 20.5$, $P < 0.001$; spaced, when: $\chi^2_2 = 6.29$, $P = 0.012$). The long-term memory for the CS was evaluated by comparing the response at 10 min to the responses at 24 h. Whether or not the bees exhibited a long-term olfactory memory depended on the sugar used as the US and the task. For this reason, we further split the analysis for each task by sugar. When sucrose was the US, massed conditioned bees were less likely to recognize the CS odour 24 h later (Fig. 3A, when: $\chi^2_1 = 8.30$, $P = 0.004$), whereas those fed with sucrose in the spaced conditioning task did not exhibit a significant drop in the response to the CS (Fig. 3B, when: $\chi^2_1 = 0.38$, $P = 0.537$). Bees fed with fructose did not exhibit robust long-term memory: in both conditioning tasks, these bees were less likely to respond to the CS during the 24 h test (Fig. 3C–D, massed, when: $\chi^2_1 = 5.78$, $P = 0.016$; spaced, when: $\chi^2_1 = 5.05$, $P = 0.025$). In contrast, bees fed with glucose had a strong memory for the CS, as these bees responded to the CS regardless of the task (Fig. 3E–F, massed, when: $\chi^2_1 = 1.16$, $P = 0.281$; spaced, when: $\chi^2_1 = 2.48$, $P = 0.115$).

We also examined the specificity of the memories formed towards the CS for bees conditioned in each task with each sugar (Fig. S3). In general, bees fed with glucose during conditioning became more specific in their responses to the CS. Bees fed with sucrose had a strong response to both odours, and bees fed with fructose were less likely to respond to both odours.

3.4. Haemolymph sugars rise 5–40 fold within 150 s after feeding

To identify how haemolymph sugars change during conditioning, we fed starved honeybees with a 5 μ l droplet of 1.0 M sucrose, glucose, or fructose and measured sucrose, glucose, fructose, and trehalose at intervals starting 30 s after feeding (Fig. 4, note ‘0’ denotes animals in the starved state). Bees fed with sucrose exhibited a change in haemolymph fructose and glucose within 1 min of feeding, whereas those fed with fructose (Fig. 4B) or glucose (Fig. 4C) had changes in haemolymph sugars that occurred within 30 s of feeding. The change in haemolymph sugars depended on the type of sugar fed to the bees (Fig. 4, Table S6, MANOVA, sugarfed \times time: sucrose: $F_{12, 189} = 2.70$, $P = 0.002$; fructose: $F_{12, 189} = 2.61$, $P = 0.003$; glucose: $F_{12, 189} = 1.67$, $P = 0.074$; trehalose: $F_{12, 189} = 1.56$, $P = 0.105$).

The flux in haemolymph glucose rose within 30 s of feeding and remained elevated up to 3–5 min afterwards. This time course of glucose flux was observed in bees fed fructose (Fig. 4B) as well as glucose and sucrose (Fig. 4, Table S6, MANOVA, time: $F_{6, 189} = 4.03$, $P = 0.001$), but it was greater in bees fed with sucrose (Fig. 4A) and glucose (Fig. 4C, MANOVA, sugarfed: $F_{2, 189} = 10.7$, $P < 0.001$; lsd *post hoc*, suc vs fruct: $P = 0.002$, gluc vs fruct: $P < 0.001$, suc vs gluc: $P = 0.168$). Haemolymph levels of fructose increased for bees fed with either sucrose (Fig. 4A) or fructose (Fig. 4B), but did not change in bees fed with glucose (Fig. 4C, lsd *post hoc*, suc vs fruct: $P = 0.429$, suc vs gluc: $P < 0.001$, gluc vs fruct: $P < 0.001$). On average, haemolymph levels of sucrose were very low or near to baseline measurements (as might be expected) with the exception of a small spike in haemolymph sucrose that was observed 3 min post-feeding for bees fed with sucrose (Fig. 4A). (Note: see Fig. S3, bees fed with a larger volume of sucrose exhibit a rapid rise in haemolymph to 200 mM sucrose at 30 s that

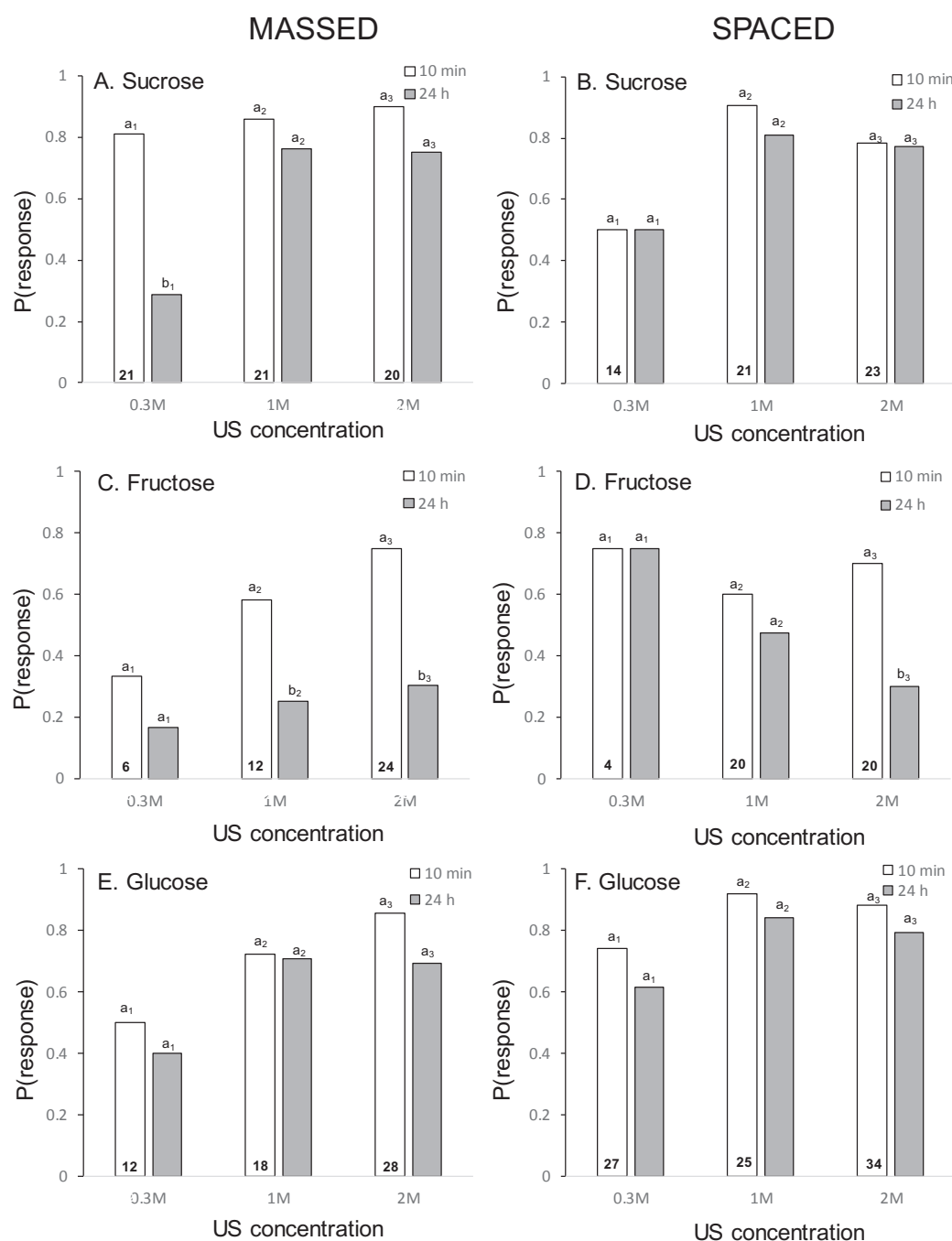


Fig. 3. Sugar quality affected long-term memory performance. A-B. Memory formation depended on reward concentration for bees fed with sucrose in a massed (A) or spaced (B) conditioning task. C-D. Bees rewarded with fructose did not form olfactory memories that lasted 24 h. E-F. Bees rewarded with glucose formed long-term memories, regardless of reward concentration and conditioning task. Letter differences indicate a pairwise-comparison with $P < 0.05$; subscripts refer to comparisons made within a specific treatment. Numbers on each bar indicate sample size per group.

decays within 3 min post-feeding to > 40 mM). Likewise, haemolymph trehalose did not vary over time after feeding (Table S6, MANOVA, time: $F_{6, 189} = 1.26$, $P = 0.278$), but the average concentration of trehalose was greater in the bees fed sucrose (MANOVA, sugarfed: $F_{2, 189} = 8.31$, $P < 0.001$; lsd *post hoc*, suc vs fruct: $P = 0.005$, suc vs gluc: $P < 0.001$, gluc vs fruct: $P = 0.275$).

4. Discussion

Our previous research established that bees formed a lasting olfactory memory when they were fed a reward during conditioning of the PER (Wright et al., 2007). Work in *Drosophila* confirmed that food rewards eaten during conditioning must be metabolically valuable to produce long-term memories (Burke and Waddell, 2011; Musso et al., 2015; Perisse et al., 2013). Our present set of experiments now reveals a

new twist in this tale: unlike *Drosophila*, bees fed with fructose during conditioning did not have robust long-term memories of the conditioned odour. Sucrose was the most phagostimulatory sugar to honeybees, and bees rewarded with sucrose were more likely to learn to associate an odour with food. However, glucose and fructose were equally phagostimulatory but bees fed with glucose were more likely to learn and to remember. By comparing learning performance to the time course of haemolymph flux in sugars, our data also indicate that memories are more robustly formed when gustatory input arrives during a period when haemolymph sugars are changing. Taken together with previous studies of food nutritional value and olfactory learning in *Drosophila*, our data suggest that the rapid change in haemolymph glucose that occurs after feeding may act as a post-ingestive signal of food value that is detected by the brain to influence learning and memory.

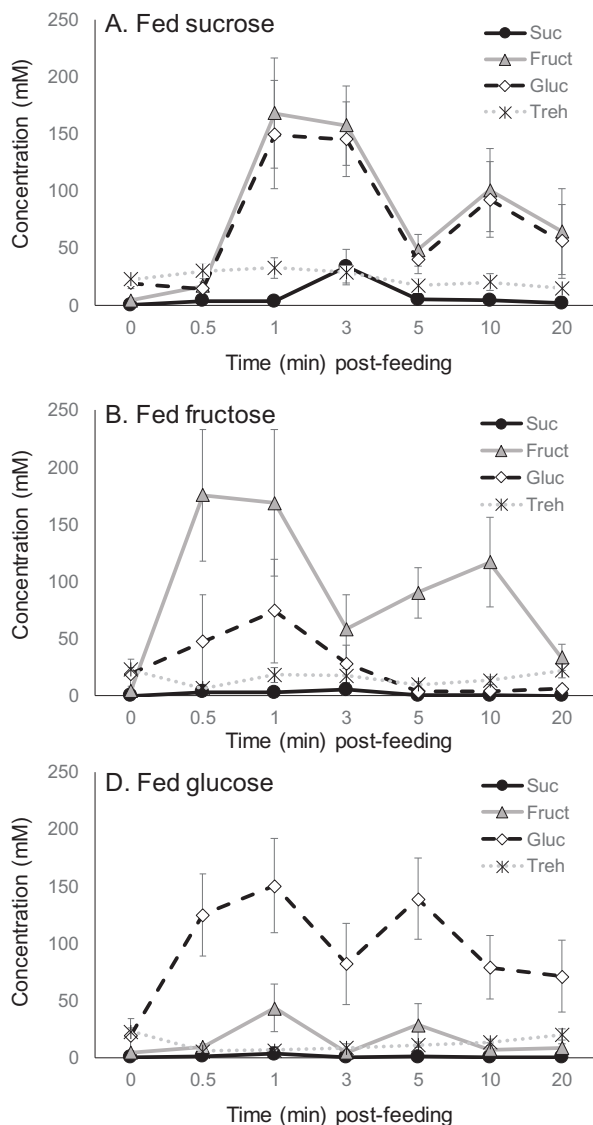


Fig. 4. Bees fed with 5 μ l fructose or glucose experience a rapid increase in haemolymph glucose and/or fructose from 30 s after feeding and within 1 min after feeding with sucrose. Concentrations (mM) \pm SEM of haemolymph sugars (sucrose, glucose, fructose and trehalose) at specified time points (30 s, 1 min, 3 min, 5 min, 10 min, 20 min) post-feeding, time point zero indicates bees that received no food. Haemolymph was extracted for 2 min at the indicated time point after the completion of feeding. Bees were fed 5 μ l of A. 1.0 M sucrose ($N = 10$ per time point), B. 1.0 M fructose ($N = 10$ per time point). C. 1.0 M glucose ($N = 10$ per time point). For all three fed sugars, haemolymph glucose and/or fructose remained elevated up to 20–70 mM at 20 min after feeding.

Of the three sugars we used as stimuli (sucrose, fructose, and glucose), sucrose has the greatest metabolic value per unit weight to a foraging honeybee. It is also a major constituent of floral nectar and is preferred by bees in free-flight tests of sugar preference (Wykes, 1952). For this reason, it is not surprising that sucrose was the strongest phagostimulant to elicit PER or feeding when contacted to the honeybee's antennae or proboscis in our experiments. Recordings from the galeal sensilla of the honeybee's mouthparts also confirm that gustatory neurons in this location have a lower detection threshold and respond with a higher rate of spiking than either fructose or glucose (Whitehead and Larsen, 1976). As predicted by learning theory (Rescorla and Wagner, 1972), we found that a high concentration US was more likely to be learned and learned more quickly. There were two cases, however, where the US concentration did not affect whether or not bees learned the task: massed learning with a sucrose US and spaced learning

with a glucose US.

The massed learning results with sucrose are consistent with a previous report comparing massed and spaced learning using sucrose in honeybees (Menzel et al., 2001). This group found that US concentration had little effect on whether or not bees learned the massed conditioning task, but a very strong effect on bees in the spaced learning task. Our data show that even very low concentrations of sucrose (0.3 M) are still strong phagostimulants to the proboscis of the bee, as $\sim 80\%$ of the subjects in our study consumed a droplet of 0.3 M sucrose in the gustatory test. A strong phagostimulant is likely to produce greater sensitization (Hammer et al., 1994). In our experiments, bees massed-conditioned with sucrose probably remain sensitized to any stimulus within 30 s of application of the US. If this is true, then the subjects fed with sucrose would still be sensitized to the stimulus after the first conditioning trial for all subsequent trials. This change in state caused by a sensitizing stimulus could make it easier for gustatory signals to form learned representations in the bee brain (Hammer et al., 1994).

The performance of bees in the spaced learning task fed with a glucose US was strong regardless of US concentration; this is not likely to be a result of sensitization, as 0.3 M glucose did not evoke a strong response from the mouthparts or antennae. Instead, we predict that glucose improved the performance of bees in the spaced learning task because it was readily mobilized into the haemolymph. Our measurements of haemolymph sugars show that glucose flux occurs within 30 s of feeding a bee with 5 μ l of a solution. Bees that experienced spaced conditioning received a reward every 5 min. Given how quickly glucose entered the haemolymph of starved (and highly motivated) bees, this suggests that the change in haemolymph glucose that occurred just after a conditioning trial influenced the strength of the CS-US association. While ingestion of sucrose also produced a haemolymph glucose flux, this flux took longer to realize, presumably because of the extra time needed for the enzymatic breakdown of sucrose into glucose and fructose. This difference in time from ingestion to peak of haemolymph concentration could be as long as 5 min, as seen in the data for bees fed to satiety with sucrose (Supplemental Fig. S3).

In contrast to action potentials from gustatory neurons that reach the brain within 30–100 ms of contact with food (Getting, 1971; Reiter et al., 2015), a post-ingestive signal caused by a change in haemolymph sugar takes more time, as food must first be consumed, digested, and pass across the gut to access the brain. In hungry bees, our data show that sugars pass across the gut into the haemolymph relatively quickly for monosaccharides, as the time needed for a sugars to reach the head capsule was 30–150 s (i.e. 0.5–2.5 min) after food ingestion. Given the speed of its incorporation into haemolymph, it is possible that the sugar itself acts as the post-ingestive signal without the need for a peptide or other signalling molecule (Burke and Waddell, 2011). Our data indicate that haemolymph glucose flux in the range of 20–170 mM experienced during conditioning acts a signal of the value of food. This change was maintained for up to 20 min after 5 μ l of food ingestion and remained even higher when bees were fed to satiety. The time course of this measurement is consistent with a previous report from honeybees in which both glucose and fructose peaked in the haemolymph collected from the abdomen within a 5 min measurement after feeding with sucrose (Crailsheim, 1988).

Studies with adult *Drosophila* have found that fructose and glucose are effective reinforcers of olfactory conditioned stimuli (Burke and Waddell, 2011; Musso et al., 2015), but no one knows exactly what that signal is. Our data indicate that the post-ingestive flux in haemolymph glucose could be the change that the brain uses to detect that a metabolic reward was eaten. In our experiments, fructose-fed honeybees had more difficulty in learning the CS-US association. While fructose was not as phagostimulatory as sucrose, most of the bees in the gustatory assays would consume > 1 M fructose solutions. Bees rewarded with 2 M fructose performed the learning task at a level comparable to glucose and sucrose-rewarded bees. As a post-ingestive signal, fructose

has the potential to be a fast signal of food value; like glucose, it entered the haemolymph quickly after starved bees consumed it. However, bees reinforced with fructose during learning exhibited poor performance in the 24 h memory test. For this reason, we conclude that fructose is not as effective as glucose at producing long-term olfactory memories in honeybees.

If glucose flux in haemolymph is the post-ingestive signal of metabolic reward, then it must pass the blood-brain barrier to interact with the neurons involved in learning and memory (see Volkenhoff et al., 2017, this issue). This flux could be detected by gustatory receptors expressed in neurons or glia in the brain (Miyamoto et al., 2012) that interact with the neurons that encode rewarding memories (Burke et al., 2012; Musso et al., 2015). Alternatively, the circuit that processes olfactory information could sense intracellular levels of ATP that arise due to intracellular nutrient flux caused by nutrient transport (Dus et al., 2013; Levin et al., 2004; Musso et al., 2015). Future studies that pinpoint specific metabolic pathways sensitive to glucose within specific neurons or circuits in the brain responsible for encoding long-term memories will uncover a fundamental property of the brain's neural circuits.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jinsphys.2017.08.009>.

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