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Olfactory memory formation and the influence of reward pathway during appetitive learning by honey bees

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Summary

Animals possess the ability to assess food quality *via* taste and *via* changes in state that occur after ingestion. Here, we investigate the extent to which a honey bee's ability to assess food quality affected the formation of association with an odor stimulus and the retention of olfactory memories associated with reward. We used three different conditioning protocols in which the unconditioned stimulus (food) was delivered as sucrose stimulation to the proboscis (mouthparts), the antennae or to both proboscis and antennae. All means of delivery of the unconditioned stimulus produced robust associative conditioning with an odor. However, the memory of a conditioned odor decayed at a significantly greater rate for subjects experiencing antennal-only stimulation after either multiple- or single-trial conditioning. Finally, to test whether the act of feeding on a reward containing sucrose during conditioning affected olfactory memory formation, we conditioned honey bees to associate an odor with antennal stimulation with sucrose followed by feeding on a water droplet. We observed that a honey bee's ability to recall the conditioned odor was not significantly different from that of subjects conditioned with an antennal-only sucrose stimulus. Our results show that stimulation of the sensory receptors on the proboscis and/or ingestion of the sucrose reward during appetitive olfactory conditioning are necessary for long-term memory formation.

Key words: *Apis mellifera*, associative learning, memory consolidation, post-ingestive feedback, gustation.

Introduction

Associative learning allows animals to predict important events using correlations between the appearance of a signal and a salient outcome such as food or danger. In classical appetitive learning, animals associate a conditioned stimulus (CS), such as an auditory tone, with an unconditioned stimulus (US) such as food (Pavlov, 1927). The strength or intensity of both the CS and the US influence the rate of learning and the duration of the memory of the association (Rescorla and Holland, 1982; Mackintosh, 1983). In the case of an appetitive US, changes in intensity (e.g. the sucrose concentration in a reward) often translate directly into a reward's nutritional value. In a natural setting, learning how to predict the occurrence of the most nutritionally valuable reward is likely to convey increased fitness. An important aspect of appetitive learning, therefore, is the evaluation of food quality either *via* pre-ingestive mechanisms such as gustatory receptors at the sensory periphery or using post-ingestive mechanisms for assessing food quality after food has been consumed.

Foraging worker honey bees learn to associate floral shapes, colors and odors with the quality and quantity of food rewards. In the laboratory, restrained honey bees will also learn to associate such cues with food rewards, especially floral odors (Bitterman et al., 1983; Frings, 1944; Kuwabara, 1957; Takeda, 1961). Experiments designed to study olfactory conditioning of honey bees have typically involved the presentation of a discrete odor CS to the antenna followed by a sucrose solution reward (US) presented first to the taste receptors on the antenna to elicit proboscis extension (the unconditioned response) and then to the proboscis so that the subject can consume the reward. Honey bees have gustatory receptors on their antennae, proboscis (mouthparts) and tarsi; stimulation of the gustatory receptors on the antennae produces the 'proboscis extension reflex' (PER) in which a honey bee will extend its proboscis expecting food (Kuwubara, 1957). Upon stimulation, the probability of eliciting proboscis extension varies directly as a function of sucrose concentration (Braun and Bicker, 1992; Scheiner, 2004; Haupt and Klemt, 2005). Sensitivity at the periphery is also modulated by a honey bee's genetic background (Page et al., 1998), age (Scheiner, 2004) and motivational state (Ben-Shahar and Robinson, 2001). When honey bees are conditioned in the classical olfactory conditioning paradigm, they are allowed to feed on the reward solution during each trial. Consuming the reward on each trial

may also provide them with the opportunity to use post-ingestive information to assess reward quality.

It is not clear whether the consumption of the food reward is a necessary condition of appetitive learning in honey bees. If an association between an odor and a sucrose reward can be formed in the absence of food consumption, this suggests that post-ingestive feedback about food quality is not necessary for appetitive olfactory learning. When a honey bee forages, it collects nectar in its crop and brings the nectar to its hive, where the nectar is stored for consumption by all colony members. During foraging, a honey bee uses the nectar it collects as food for itself, but the passage of nectar from the crop to the midgut is optimized such that as much nectar as possible is returned and regurgitated as food for the colony (Blatt and Roces, 2001). Honey bees, therefore, may not rely on post-ingestive feedback about nectar quality since they do not forage for their own immediate benefit and may not eat most of the food they collect. Indeed, in their definitive series of experiments, Bitterman et al. observed that it was possible for honey bees to learn to associate an odor stimulus with sucrose delivered to the antennae without the subject being allowed to consume the reward (Bitterman et al., 1983). However, the strength and duration of the memory formed *via* antennal-only or proboscis-only stimulation has not been investigated.

The purpose of the present study is to investigate the extent to which the reward pathway experienced during olfactory learning affects the formation of memory in honey bees. In our experiments, we examined in detail whether stimulation of a honey bee's antennae is sufficient to allow robust associative conditioning, short-term memory formation and long-term memory formation. To do this, we conditioned honey bees by stimulation of the antennae alone, stimulation of the proboscis followed by feeding or stimulation of both the antennae and the proboscis with feeding and then tested them for their responses to the conditioned odor immediately, 24 h and 96 h after conditioning. Furthermore, because pollen foragers are more sensitive to sucrose than nectar foragers (Page et al., 1998), we also examined whether pollen foragers learned faster and had a longer memory of the conditioned odor than nectar foragers. Finally, by feeding our subjects a water reward, we examined whether the presence of sucrose in the ingested reward was necessary for formation of long-term odor memory.

Materials and methods

General methods

Subjects

Worker caste New World Carniolan honey bees (*Apis mellifera*) were collected from colonies maintained at the Rothenbuhler Honey Bee Research Laboratory at Ohio State University or at the School of Life Sciences at Arizona State University. Subjects for Experiments 1 and 2 were collected between August and October 2004 at Ohio State University, and honey bees used in Experiments 3 and 4 were collected in March 2007 at Arizona State University. Individual pollen- and nectar-foraging workers were collected from several different outdoor hives. A wire mesh excluder was placed over the hive entrance to facilitate the capture of individuals returning from foraging before they entered the hive. Pollen foragers were easily identified by balls of pollen in the corbiculae of the hind legs. Foragers without pollen were assumed to be nectar foragers. We chose to use this method of identifying pollen and nectar foragers because it did not require forcing each bee to regurgitate its crop contents, which is potentially damaging to our subjects and could interfere with their performance in the experiments. We did, however, collect crop regurgitant from a subset of the nectar-foraging subjects. Crop samples were taken by placing a subject on its dorsal surface and squeezing its abdomen, causing the forager to regurgitate its crop contents into a 50 μl microcapillary tube held against the mandibles. These individuals were discarded after the nectar sample was collected. The sucrose concentration of the nectar was then assessed using a Leica BRIX refractometer (N=126). Approximately 50% of these subjects did not have any crop contents when forced to regurgitate. Of the subjects with nectar in their crop, 12% of these were water foragers, and the other 88% had nectar with a median sugar concentration of 32%.

Individuals were captured in small vials, placed on ice until they ceased moving and then secured in a restraining harness by a strip of tape between the head and the thorax, allowing free movement of the antennae and proboscis (mouthparts). Each subject was fed 1.5 mol l^{-1} sucrose until satiated and held for ~24 h before conditioning. Immediately prior to the experiments, each honey bee was evaluated for motivation by lightly touching one antenna with the sucrose solution without subsequent feeding. If a subject responded by extending its proboscis (PER), it was selected for use in the experiment.

Odors

The odor stimuli used during olfactory conditioning in our experiments were 1-hexanol and 2-octanone (98% purity; Sigma-Aldrich, St Louis, MO, USA) diluted in hexane to 2.0 mol l^{-1}. A 5 μl aliquot of odor solution was placed onto a small strip of filter paper placed in a modified 1 ml tuberculin glass syringe attached to an air source that was controlled by a 2-way valve connected to a Programmable Logic Controller (Automation Direct, Cumming, GA, USA) for precise stimulus delivery (Wright and Smith, 2004). Odor stimuli were counterbalanced throughout experiments and treatments.

Conditioning protocols

Three different associative conditioning regimes were used in which the presentation of the unconditioned stimulus (US) varied: antennal-only conditioning (AC), proboscis-only conditioning (PC) and antennal-plus-proboscis conditioning (APC). The APC protocol is the 'classical' conditioning paradigm as described in Bitterman et al. (Bitterman et al., 1983). For each type of conditioning, subjects receive stimulation with an odor conditioning stimulus (CS) that is paired with a closely timed appetitive reward. In our experiments, an odor (CS) was presented in a discrete air pulse for 4 s. The reward (US), a 0.4 μl droplet of a 1.5 mol l^{-1} sucrose solution, was presented 3 s after the start of the odor so that presentation of the CS and US overlapped for 1 s. In the APC protocol, the sucrose solution was first presented to the antennae, eliciting proboscis extension, and then to the proboscis such that the subject consumed the entire droplet. This was performed on each trial, even if the subject extended its proboscis in response to the odor. The AC protocol differed from the APC protocol in that only the antennae were

stimulated with sucrose; the subject was not allowed to touch the sucrose solution with its proboscis or to feed during conditioning. The PC protocol was different from APC in that the antennae were not stimulated with sucrose solution to elicit proboscis extension during conditioning. In this case, the sucrose solution was applied directly to the proboscis such that the entire droplet was consumed on each trial without contacting the antennae. Each subject received conditioning trials at an inter-trial interval of 5 min. During conditioning with each protocol, if a subject had learned to associate the CS with the US, then proboscis extension would occur before presentation of the US. Thus, a learned response was scored as a binary variable (response or no response). After conditioning, each subject was tested for recall of the association either immediately (5 min after the last acquisition trial), 24 h or 96 h after conditioning. Each subject was tested for recall at only one time point. During the recall tests, the odor stimuli were presented without sucrose reinforcement at the same inter-trial interval used during conditioning. The presence or absence of a response was recorded.

Experiment 1. Comparison of acquisition and recall in the AC, PC and APC protocols for pollen and nectar foragers

The first series of experiments examined how the conditioning protocol (AC, PC, APC) affected the level of associative conditioning and recall for the CS. All subjects experienced 16 acquisition trials in a pseudo-randomized sequence (e.g. A-B-B-A-B-A-A-B-A-B-B-A-B-A-A-B, where A denotes a reinforced conditioning trial and B denotes a trial in which another odor was experienced without reinforcement). Eight recall test trials followed the acquisition phase. Prior to the recall test trials, honey bees were tested for motivation as described above; only subjects that responded to antennal stimulation were used. During the recall tests, the reinforced odor from the A trials or the unreinforced odor from the B trials was presented in the following sequence of trials, A-B-B-A-B-A-A-B, and the response was recorded. No sucrose stimulation was presented during the recall tests. Recall test trials occurred at one of three time points after conditioning: immediately, 24 h or 96 h. The subjects tested at either the 24 or 96 h time points were fed 1.5 mol l^{-1} sucrose solution following conditioning, until satiated, and were then held in a humidified box until the recall tests were performed. Subjects tested at the 96 h time point were additionally fed to satiety every 24 h.

Experiment 2. Test of associative conditioning for the AC protocol

We performed a pairing control experiment with antennal stimulation to evaluate whether conditioned responding (proboscis extension) to the odor (CS) arose due to non-associative (sensitization) or to associative conditioning. Subjects, exclusively pollen foragers, were assigned to one of three protocols – forward pairing, backward pairing or unpaired – and experienced 16 acquisition trials in the pseudo-randomized sequence A-B-B-A-B-A-A-B-A-B-B-A-B-A-A-B described before. In the forward pairing treatment, the A trials consisted of odor (CS) followed by sucrose stimulation (US) as described in Experiment 1. For the B trials, the subject was placed in the conditioning arena, but no odor or sucrose was delivered. In the backward pairing protocol, the A trials were performed such that the sucrose solution (US) was presented to the antennae 3 s before the presentation of the 4 s odor (CS). For the B trials, the subject was placed in the conditioning arena, but no odor or sucrose was delivered. In the unpaired protocol, the A trials consisted of stimulation with the odor only (CS) whereas B trials consisted of presentation with just sucrose (US). In all three protocols, after conditioning, two recall test trials were performed by presenting the CS without the US and the responses were recorded.

Experiment 3. Comparison of recall after a single conditioning trial in the AP, PC, APC and unpaired protocols

This experiment was designed to examine the extent to which the memory of the CS odor was formed after one trial of conditioning with the APC, PC or AC protocols. Subjects received two placement trials in either the paired or unpaired treatment. For the paired treatment, the odor (CS) was presented approximately 3 s before the sucrose (US) (as described in Experiment 1) for one conditioning trial. The second trial was placement of the subject in the conditioning arena only. For the unpaired treatment, the odor (CS) was presented on one trial and the sucrose (US) was presented on the other trial. The order (trial 1 or trial 2) of the presentation of sucrose or odor was randomized across trials. After conditioning, each subject was given four test trials with the odor at one of three time points: immediately, 24 h or 96 h. As described in Experiment 1, subjects tested at the 24 or 96 h time points were fed to satiation every 24 h to prevent starvation.

Experiment 4. The role of sucrose consumption in memory formation

Relatively higher levels of conditioned responding by subjects in the APC and PC protocol groups could reflect the proboscis (feeding-related) components that were present in those protocols but not in the AC protocol. In the APC and PC protocols, at least two processes may influence the CS–US association. First, there are multiple mechano- and hygro-sensory components that arise from feeding movements and water uptake during ingestion. Second, taste receptors on the proboscis may respond to sucrose in the solution. This experiment was designed to test whether the stimulation of the proboscis with sucrose and/or ingestion of sucrose were necessary for the retention of CS memory. Pollen foragers were conditioned for eight rewarded trials using one of three protocols. Two of the protocols (APC, AC) were the same as described in Experiment 1. In addition, a new protocol (ACW) was used in this experiment: the antennae were stimulated with sucrose solution to elicit proboscis extension, but a 0.4 μl droplet of water was applied to the proboscis instead of the sucrose solution. The water was presented to the proboscis and consumed by the subjects as in the APC protocol. Subjects were tested for recall immediately or 24 h after training. As in the other experiments, subjects tested at 24 h were fed to satiation after training.

Statistical analysis

For all of the behavioral experiments, the responses of subjects were scored as binary variables. Using the SAS

statistical software, we used repeated-measures logistic regression (rpm lreg) or logistic regression (lreg) with least-squares *post hoc* contrasts (lsc) for multiple comparisons to test all hypotheses (Agresti, 1996). This method is similar to analysis of variance, in that it allows for the construction of models to test the effects of experimental parameters on a behavioral outcome; logistic regression was developed for testing hypotheses when the dependent variable is scored as either a 0 or 1. In the figures, means are reported as probabilities of responding along with estimated standard errors of this probability (Cox and Snell, 1989).

Results

Experiment 1A. Conditioning protocol and foraging phenotype affect olfactory learning

For each conditioning protocol, the responses to the reinforced odor A were significantly higher than those to the unreinforced odor B (Fig. 1) (rpm lreg; χ_1^2=96.4, P<0.001). In fact, all three treatment groups reveal a pattern of responding to A relative to B that is typical for associative differential conditioning (Bitterman et al., 1983). Initial response levels to odor A were low and increased rapidly to reach an asymptote by approximately the 4th or 5th trial. The initial response to the unreinforced odor B was initially higher than that to odor A, because the first trial with B always followed the first trial with A. This higher initial response was probably due to sensitization from the preceding A trial and/or to excitatory generalization from A to B. However, the response to B never increased beyond this and in fact remained lower than A in the 2nd–8th trials.

Although each conditioning protocol produced an association, the rate of acquisition of the conditioned response on the reinforced A trials depended on the protocol (APC, PC, AC) used during conditioning (Fig. 1) (3-way interaction, rpm lreg; χ_2^2=8.27, P=0.016). Honey bees conditioned *via* the APC protocol (Fig. 1A), in which they experienced stimulation on both the antennae and the proboscis, had a greater rate of acquisition than those trained with either the PC (proboscis stimulation only) (Fig. 1B) (lsc; APC *vs* PC; χ_1^2=5.39, P=0.020) or AC (antennal stimulation only) (Fig. 1C) (APC *vs* AC; χ_1^2=9.99, P=0.002) conditions. For example, on trial 2, approximately 30% of bees responded to the odor in the APC protocol, and this percentage increased to 40–60% on trial 3. The corresponding percent response on the same trials was much lower in PC and AC stimulation groups, and these differences were carried through the remaining trials. The rate of acquisition was not significantly different for the PC and AC protocols (AC *vs* PC; χ_1^2=0.60, P=0.436).

We expected that pollen foragers would have a higher sensitivity to sucrose and would, therefore, also have a greater ability to learn to associate an olfactory stimulus with a food reward. Pollen foragers, however, did not consistently display greater asymptotic levels of association of odor with a sucrose reward than nectar foragers. Their learning abilities varied as a function of the conditioning protocol. For the APC protocol, the asymptotic level of acquisition was greater for the pollen than for the nectar foragers (rpm lreg; χ_1^2=4.21, P=0.040). By contrast, with the PC protocol, the asymptotic level of acquisition was greater for nectar foragers than for pollen foragers (χ_1^2=5.17, P=0.020). For the AC protocol, no difference in the final level of acquisition was observed between pollen and nectar foragers (rpm lreg; χ_1^2=0.18, P=0.671).

Experiment 1B. Recall of an olfactory stimulus depends on conditioning protocol, time post-conditioning and foraging phenotype

To examine how conditioning protocol affected olfactory memory formation, we tested the ability of our subjects conditioned with each protocol (APC, PC, AC) to recall the

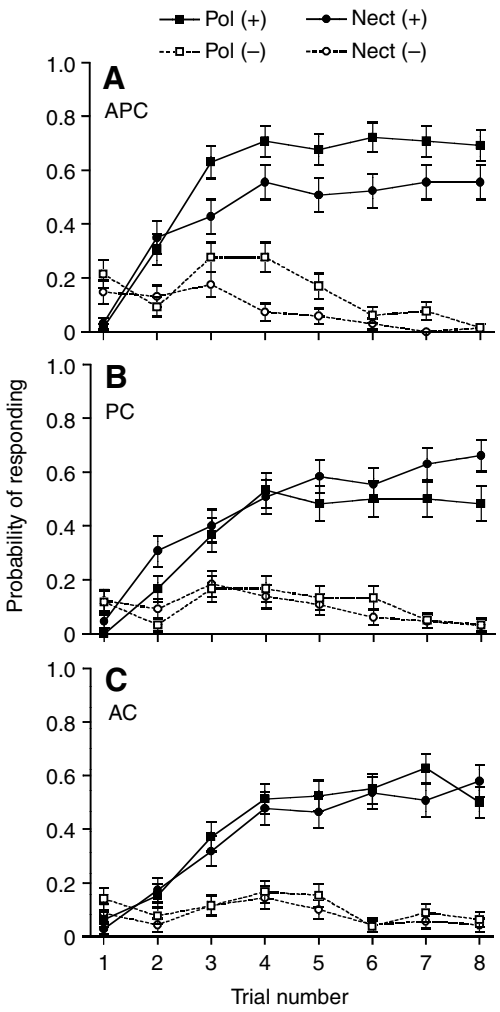

Fig. 1. Rates of acquisition for honey bees trained with three different conditioning protocols reflected in the mean (± s.e.m.) response on each trial. (A) APC protocol where both antennae and proboscis are stimulated with sucrose solution and the bees are allowed to consume the sucrose reward (N_{pollen}=65; N_{nectar}=63). (B) PC protocol where only the proboscis is stimulated and bees are then allowed to consume the reward (N_{pollen}=78; N_{nectar}=69). (C) AC protocol where the antennae are stimulated with sucrose solution, but subjects were not allowed to touch the sucrose solution with their proboscis or consume the reward (N_{pollen}=60; N_{nectar}=65). The rate of acquisition was greatest for the APC protocol; honey bees conditioned using the PC or AC protocols did not have significantly different rates of acquisition. The predicted probability of responding on each conditioning trial is shown with the ± s.e.m. of this probability. Abbreviations: Pol, pollen foragers; Nect, nectar foragers; +, reinforced trials with CS odor on the A trials; –, B trials with the unreinforced odor.

conditioned odor during a unreinforced test administered immediately, 24 h or 96 h after conditioning. The response on the first test trial to the conditioned odor was used to assess recall. We observed that the probability that a honey bee responded during the test depended on forager type, conditioning type and the post-conditioning time of the test (Fig. 2) (3-way lreg; χ_4^2=12.1, P=0.017). Because the responses were different for pollen and nectar foragers, separate analyses were performed on each foraging phenotype. For pollen foragers, the probability of responding to odor during the test depended on both the conditioning protocol and the time post-conditioning (Fig. 2A) (2-way lreg; χ_4^2=13.4, P=0.009). Immediately after conditioning, subjects responded strongly to the conditioned odor. However, the probability of responding during the test dropped significantly after 24 and 96 h, with the most precipitous drop occurring in the first 24 h on average (Fig. 2A). This decrease is consistent with decay of memory as it consolidates through different phases. Immediately after conditioning, subjects in the APC, PC and AC groups responded equally well to the conditioned odor. However, 24 and 96 h after conditioning, the responses of subjects conditioned with the AC protocol were significantly lower than those of subjects conditioned with the APC or PC protocols. Therefore, we conclude that the conditioning protocols produced differences in the extent to which long-term memory was consolidated. Antennal-only stimulation supported immediate recall, but it was less capable of supporting consolidation into long-term memory.

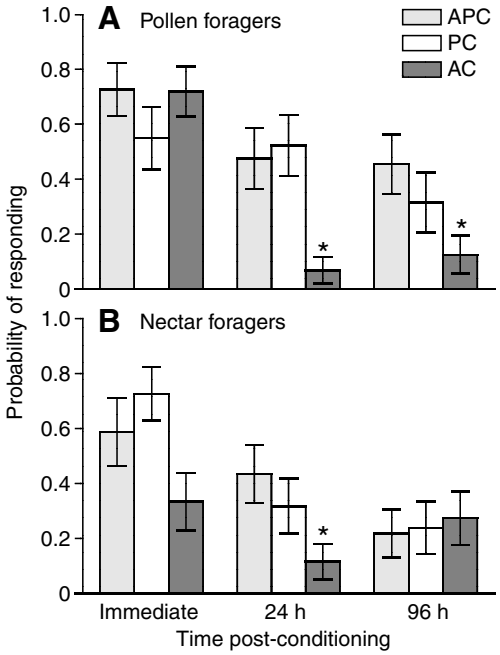

Fig. 2. Recall is greatest immediately after conditioning, and the rate of memory decay depends on the protocol used for conditioning. The proportion of honey bees responding to the rewarded odor during the first recall test trial at different times after conditioning is shown. (A) Pollen foragers were conditioned using the APC, PC or AC regime and then tested for recall immediately (N_{APC}=22; N_{AC}=22; N_{PC}=21), after 24 h (N_{APC}=25; N_{AC}=29; N_{PC}=24) or 96 h (N_{APC}=20; N_{AC}=21; N_{PC}=19). (B) Nectar foragers were conditioned using one of the three training protocols and were then tested for recall immediately (N_{APC}=17; N_{AC}=23; N_{PC}=23) or 24 h later (N_{APC}=21; N_{AC}=26; N_{PC}=22) or 96 h later (N_{APC}=22; N_{AC}=22; N_{PC}=21). Recall for AC and PC was compared to that for APC using a least-squares contrast at each time point; * indicates a difference of P<0.05.

For nectar foragers (Fig. 2B), as with pollen foragers, the level of response on the first test trial depended on the time post-conditioning (lreg; χ_1^2=31.2, P<0.001), with a decline in response levels starting 24 h after conditioning. On average, the APC and PC conditioned subjects also displayed a greater probability of responding to the test odor than subjects conditioned with the AC protocol (lreg; χ_2^2=6.64, P=0.024). For example, nectar foragers conditioned with the AC protocol responded significantly less to the conditioned odor 24 h after conditioning than the APC and PC subjects. By 96 h after conditioning, however, the probability of a subject responding to the conditioned odor during the test was equal for all three conditioning protocols.

Experiment 1C. Extinction is equal across foraging phenotype, conditioning protocol and time post-conditioning

By measuring responses during eight unreinforced recall test trials with the odor A (reinforced CS) and odor B (unreinforced CS), we examined both the rate of extinction of the response and the effect of time after conditioning (immediate, 24 h or 96 h) on the rate of extinction. The response to the reinforced CS odor (A) was significantly greater during the test trials than the response to the unreinforced odor (B) for all treatments (rpm lreg; χ_1^2=151, P<0.001). The responses of all subjects during the test showed extinction, as the probability of responding decreased as a function of trial number (rpm lreg; χ_1^2=31.2, P<0.001). The rate of extinction of the conditioned response to the CS (A trials) as a function of trial number, however, was not significantly different for any of the conditioning protocols measured separately at each time post-conditioning for pollen or nectar foragers (4-way interaction, rpm lreg; χ_4^2=6.19, P=0.186). The response on the first trial was significantly greater than the response on all the other trials (for all three, P<0.001) but the probability of responding on test trials 2, 3 and 4 was not significantly different (for all, P>0.999). The largest change in the slope of extinction during the recall test trials occurred between trials 1 and 2.

Experiment 2. Antennal-only conditioning produces associative learning

Successful discrimination of the reinforced odors (A trials) from unreinforced odors (B trials) in Experiment 1 suggests that the responses observed for all three types of US stimulation during conditioning were driven by associative learning mechanisms (Mackintosh, 1983). If the responses during conditioning had been driven by non-associative mechanisms such as sensitization to the sucrose stimulation, we would have expected much higher response levels to the unreinforced odor (B trials). Previous studies (Bitterman et al., 1983; Sandoz et al., 2002) suggest that antennal stimulation paired with an olfactory CS alone could produce associative learning. We extend these studies by comparing the recall of the CS for subjects conditioned with antennal-only forward, backward or unpaired CS–US presentations. The most robust conditioning

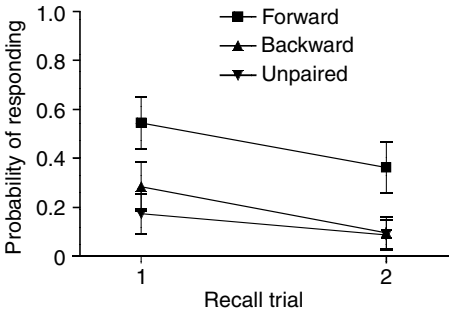

Fig. 3. An odor paired with stimulation of the antennae produces associative learning. Pollen foragers conditioned with forward pairing of the odor and sucrose stimulation of only the antennae demonstrated the most robust conditioning. Recall of honey bees receiving backward or unpaired conditioning of odor with antennal-only stimulation was significantly lower than that of the forward-paired subjects (χ_2^2=7.45, P=0.024). The proportion of bees responding to the conditioned odor during two recall tests is shown ($N_{forward}$=22; $N_{backward}$=21; $N_{unpaired}$=23).

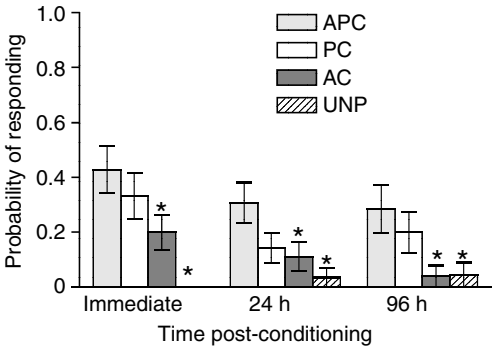

Fig. 4. Conditioning protocol also affects recall after single-trial conditioning. The proportion of subjects responding to the first recall test with the rewarded odor immediately (N_{APC}=35; N_{AC}=40; N_{PC}=33; N_{UNP}=31), 24 h (N_{APC}=39; N_{AC}=25; N_{PC}=42; N_{UNP}=29) or 96 h (N_{APC}=48; N_{AC}=75; N_{PC}=30; N_{UNP}=22) after conditioning is shown. None of the honey bees trained with the unpaired protocol responded on the immediate recall test. Recall at each time post-conditioning depended upon the conditioning protocol (lreg; χ_3^2=39.6, P<0.001). Recall of the odor CS decays for all conditioning protocols (APC, PC, AC) over a period of 96 h (lreg; χ_2^2=6.27, P=0.043). Recall for AC, PC and UNP was compared to that for APC using a least-squares contrast at each time point; * indicates a difference of P<0.05.

occurred with the forward-paired treatment (Fig. 3) (rpm lreg; χ_2^2=7.45, P=0.024). Subjects conditioned in the forward-paired treatment had a significantly higher probability of responding during the recall tests than those in either the backward-paired (P=0.024) or the unpaired (P=0.007) groups. The levels of response for honey bees conditioned with the backward or unpaired treatment were not significantly different (P=0.502). The probability of responding during the test trials was greatest on the first test trial (Fig. 3) (rpm lreg; χ_1^2=7.14, P=0.007), and the rate of extinction over the two test trials was the same regardless of the type of conditioning (2-way rpm lreg; χ_2^2=0.47, P=0.791). If responses during the recall test to the unpaired or backward-paired treatments were not significantly different from the forward-paired treatment, it is possible that the AC protocol did not produce proboscis extension in honey bees *via* associative learning of the odor (CS) with the sucrose (US). However, because responses were greatest for the forward-paired group, we conclude that the AC protocol produces associative conditioning between an odor CS and a sucrose solution US applied to the antennae.

Experiment 3. Antennal-only conditioning shows deficits in recall after one trial learning

The association formed after a single conditioning trial in olfactory learning by honey bees is currently thought to proceed from different pathways in the central nervous system than those that give rise to long-term memory formed after several conditioning trials (Eisenhardt, 2006; Schwärzel and Müller, 2006). With this in mind, we examined how the type of conditioning protocol influenced recall after a single conditioning trial using four conditioning protocols: APC, PC, AC or unpaired (UNP). As before, we used the proportion of bees responding on the first recall test trial to assess how the conditioning protocols and the time post-conditioning affected the recall of the CS odor (Fig. 4). As observed in Experiment 1, the level of response to the CS odor during the test depended upon the type of conditioning each subject received (APC, AC, PC, UNP) (lreg; χ_2^2=39.7, P<0.001) and upon the duration of time between conditioning and testing (lreg; χ_2^2=6.27, P=0.043). The response was greatest for the test that took place immediately (Immediate *vs* 24 h, P=0.003; Immediate *vs* 96 h, P=0.004), but the response at 24 h was not significantly greater than the response at 96 h (P=0.927).

As before, we also examined whether the rate of extinction of conditioned responding during the test period depended on the conditioning protocol; in this case, we only compared the APC, AC and PC protocols. We examined the proportion of subjects responding during four unreinforced test trials after one forward-paired trial. The rate of extinction across all four test trials (indicated by a significant 3-way interaction in the logistic regression model) was the same for all three conditioning protocols (APC, PC, AC) at each time post-conditioning (3-way rpm lreg; χ_6^2=7.36, P=0.118). However, the number of subjects responding across all four trials during the test decreased as a function of trial for all conditioning types (APC, AC, PC) and for all three time points tested post-conditioning (rpm lreg; χ_1^2=180, P=0.001). The response on the first trial was, on average, greater than the response on every other trial (all three lsc; P<0.001). The responses on all other trials were not significantly different (P>0.999).

Experiment 4. Sucrose reward is necessary for long-term recall

This experiment was designed to examine whether feeding on sucrose was necessary and/or sufficient for producing an increased ability to recall the olfactory CS in the APC and PC protocols. We compared individuals conditioned in the APC and AC protocols to subjects conditioned in the APW protocol, in which the antennae were touched with a sucrose solution US but the subject was fed water. The rate of acquisition, reflected in the difference in the slope of the acquisition curve for each protocol, depended upon the conditioning protocol (Fig. 5A) (2-way interaction; rpm lreg; χ_2^2=7.80, P=0.020). The rate was

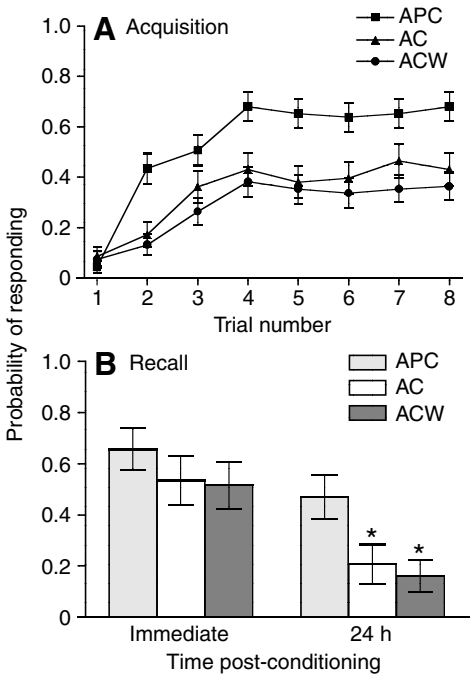

Fig. 5. The presence of sucrose in the reward strongly affects the ability of honey bees to recall the olfactory CS. Pollen foragers were conditioned with the APC protocol, the antennal-stimulation-alone (AC) protocol or a protocol where the antennae were stimulated with sucrose solution but the subject was fed water (ACW). (A) The rate of acquisition was greatest for the APC protocol (lreg; χ_2^2=7.80, P=0.040) (N_{APC}=69; N_{AC}=58; N_{ACW}=68). (B) When tested immediately after conditioning, the level of response of subjects to the CS during the first recall trial was not significantly different for the three protocols (χ_2^2=1.60, P=0.449) (N_{APC}=35; N_{AC}=29; N_{ACW}=31). After 24 h, the response of the AC and ACW subjects was significantly lower than the responses of the APC subjects (χ_2^2=9.21, P=0.010) (N_{APC}=34; N_{AC}=29; N_{ACW}=37). *P<0.05.

greatest for the APC protocol (APC *vs* AC, P=0.052; APC *vs* ACW, P=0.027) and was not significantly different for the AC and ACW protocols (P=0.841). During the recall test trial with the conditioned odor, the level of response was not significantly different at the immediate time point for the three different protocols (lreg; χ_2^2=1.60, P=0.449). However, at 24 h post-conditioning, the APC-conditioned subjects responded with a greater probability to the conditioned odor than the AC- or ACW-conditioned subjects (lreg; χ_2^2=9.21, P=0.010; lsc; APC *vs* AC, P=0.036; APC *vs* ACW, P=0.007; AC v*s* ACW, P=0.647).

Discussion

Under natural conditions, foraging honey bees process information about nectar quality through two different pre-ingestive gustatory pathways: the antennae and the proboscis. In typical olfactory classical conditioning in honey bees, both pathways are stimulated with sucrose (US) to produce a CS–US association. Furthermore, learning in both contexts involves the consumption and the possible post-ingestive evaluation of the sucrose reward. In our experiments, stimulation of either the antenna or the proboscis or both produces an association with an olfactory CS and an olfactory memory. Subjects receiving either compound stimulation (APC) or proboscis-only US stimulation (PC) formed longer-lasting memories for the CS odor than subjects conditioned with the antennal-only US. We also observed that the way in which the conditioning protocol affected retention of long-term memory for the CS depended upon whether honey bees were classified as pollen or nectar foragers.

Formation of long-term memory: evidence for pre- vs post-ingestive mechanisms

One of the most striking results of our study is that feeding on the reward, which includes both stimulation of the gustatory receptors on the proboscis and ingestion of the reward solution, strongly affected long-term memory. Subjects conditioned with an antennal-only US (AC) maintained a memory for the association between the odor CS and sucrose US for a much shorter period than honey bees that received proboscis stimulation (APC or PC). In a recent study, Scheiner et al. (Scheiner et al., 2005) observed that sensitivity to sucrose on the antenna was correlated to sucrose sensitivity on the proboscis of honey bees. Furthermore, they observed that, although honey bees showed higher sensitivity to stimulation of the antennae than the proboscis, it was the concentration of sucrose applied to the proboscis and then consumed that determined the level of acquisition. Pre-ingestive perception of the quality of the reward, therefore, affects acquisition differently depending on how the reward is experienced at both the antennal and proboscis sensory pathways. Our results confirm that information about the reward solution, as experienced at the proboscis, strongly influences acquisition. This was especially obvious in the experiment in which stimulation of the antennae with sucrose followed by feeding with water (ACW) produced levels of acquisition and memory recall equivalent to conditioning with antennal stimulation alone (AC). Our results suggest that the mechano- and hygro-sensory stimulation of the proboscis during feeding does not rescue recall. Taken together, these results establish that stimulation of the proboscis, which includes both sensory perception and/or consumption of the reward, plays a significant role in memory retention.

Gustatory information from chemosensory receptors on the antennae and the proboscis projects to different areas of the honey bee brain. Gustatory sensory neurons in the proboscis project to the subesophageal ganglion (Mitchell et al., 1999) and connect to the mushroom bodies *via* the subesophageal-calycal tract (Schröter and Menzel, 2003), whereas gustatory neurons from the antennae project to the dorsal lobe (Suzuki, 1975) and do not appear to project to the subesophageal ganglion or, indeed, to the antennal lobe (Haupt, 2007). One explanation for the differences we observed both in acquisition and in long-term memory retention may be due to the differences in the contribution of sensory information from each of these separate gustatory inputs. In particular, it is likely that the mushroom bodies support a greater capacity for associative learning and for memory consolidation of multimodal inputs. If this is true, then the greater memory retention for the subjects conditioned with the APC and PC protocols would be expected.

Our study also suggests that post-ingestive feedback about

the quality of the reward may influence the formation of long-term memory. In humans and rats, glucose levels in the brain have been shown to affect learning and memory formation and may act through a variety of mechanisms including affecting the energy available for neurons, affecting levels of neurotransmitters or by acting directly as a neuromodulator (McNay and Gold, 2002). In honey bees, post-ingestive feedback about the quality of a reward may also be conveyed by changes in hemolymph glucose or other sugar levels following sucrose consumption during and immediately after conditioning. In other insects, hemolymph sugar levels have been shown to affect the probability of feeding and gustatory sensitivity in peripheral taste cells (Simpson et al., 1990; Simpson and Simpson, 1992). Satiety, and hence motivation to feed, is also mediated by hemolymph levels of amino acids and sugars in locusts (Zanotto et al., 2002). In honey bees, the level of satiation prior to conditioning affects single as well as multiple trial learning (Friedrich et al., 2004); it also affects gustatory sensitivity and expression of the PER reflex (Pankiw et al., 2002; Pankiw et al., 2004). It is conceivable, therefore, that when food is absorbed from the midgut into the hemolymph during conditioning that hemolymph sugar levels, perhaps mediated by glucose (Crailsheim, 1988; Roces and Blatt, 1999; Blatt and Roces, 2002), provide feedback to the brain about the quality of the reward both during and after conditioning.

Recent studies of the cAMP–PKA pathway in the brain of honey bees have shown that, as with other invertebrates and vertebrates, this pathway is important in the formation of long-term memory (for reviews, see Eisenhardt, 2006; Schwärzel and Müller, 2006). Inhibition of PKA during acquisition results in significantly reduced levels of recall in multiple trial, but not single trial, learning (Müller, 2000). Olfactory learning studies in satiated honey bees have shown that satiated subjects respond at lower levels than hungry subjects during conditioning and that the brains of satiated honey bees show significantly lower levels of PKA activity (Ben-Shahar and Robinson, 2001; Friedrich et al., 2004). Furthermore, pharmacologically increasing the low PKA activity in satiated honey bees before conditioning rescued the ability of satiated honey bees to form long-term memories (Friedrich et al., 2004). Taken together, these results suggest that a feedback mechanism may exist such that the level of satiety influences PKA activity in the brain, which then affects both the acquisition and the formation of memory. Our results are consistent with this model, and furthermore suggest that the consumption of the sucrose reward provides post-ingestive feedback that may be involved in the determination of food quality and nutritional state.

Learning processes: acquisition, retention and extinction

Although previous studies have shown that honey bees could acquire an association between an odor CS and a sucrose US *via* antennal-only stimulation (Bitterman et al., 1983; Sandoz et al., 2002), our study is the first to show that the relatively high levels of response seen during antennal conditioning do not translate into robust retention of the association. The analysis of genetic mutants and the use of pharmacological tools have shown that the process of acquisition is distinct from that of recall. For example, activation of protein kinase C (PKC) in the antennal lobes has been shown to affect memory, but not acquisition, in the honey bee (Grünbaum and Müller, 1998). These studies and many others (see Schwärzel and Müller, 2006) suggest that acquisition and recall are dependent on distinct biochemical pathways.

The ability of bees to recall an association after conditioning with antennal-only stimulation showed marked deficits when compared to the recall of bees conditioned with the APC or PC protocols. After a single conditioning trial, AC-conditioned subjects exhibited a much lower response to the CS than those conditioned with the APC protocol, even when tested immediately after conditioning. When multiple conditioning trials were given, bees trained *via* antennal-only stimulation initially showed recall levels similar to those for APC and PC bees. This difference between single- and multi-trial conditioning suggests that multiple training trials may compensate for the weaker association formed by antennal-only training. However, it is clear that multiple antennal stimulation trials cannot fully compensate for the lack of proboscis stimulation and/or sucrose consumption, as the memory of multiple-trial AC-conditioned bees decayed rapidly and recall was significantly lower at 24 h after conditioning. Memories produced *via* single-trial conditioning are produced by mechanisms distinct from those producing long-term memories (reviewed in Eisenhardt, 2006; Schwärzel and Müller, 2006). However, the fact that memory retention is much reduced in AC bees conditioned with single or multiple trials suggests that reward consumption may influence multiple mechanisms underlying memory formation.

In our experiments, extinction was induced by four trials of the conditioned odor (CS) presented without reward (US). It is presently unknown whether extinction occurs *via* the same associative learning mechanisms as excitatory conditioning or whether it is a distinct form of learning. Current models of the process of extinction suggest that it is a form of learning rather than the destruction of the original association formed between the US and the CS (Bouton, 2004; Rescorla, 2004; Rescorla, 2006; Eisenhardt and Menzel, 2007). If extinction of conditioned responding proceeded from the same physiological mechanism as excitatory learning, we might expect it to compete with the memory of the original CS–US association. Based on this rationale, one might expect that a strong CS–US association would show a slower rate of extinction than a weak CS–US association. In our experiments, therefore, extinction of conditioned responding should have been slowest for the APC protocol. Although we observed that the APC protocol produced a greater rate of acquisition and a longer memory than the AC or PC protocols, the rate of extinction of the CS memory was not significantly different for the three protocols. Because CS presentation during extinction was the same for all three protocols, our data suggest that different physiological mechanisms underlie acquisition and extinction in honey bees (Eisenhardt and Menzel, 2007).

Differences between pollen and nectar foragers

In a honey bee colony, the genetic background of workers can lead to differences in foraging behavior such that some workers focus on collecting nectar while others collect pollen, pollen and nectar, or water (Fewell and Page, 1993; Page et al., 1998; Pankiw and Page, 2000). Foragers that specialize in

collecting nectar have a reduced sensitivity to sucrose compared to pollen, nectar and pollen, and water foragers when their antennae or tarsi are stimulated to elicit proboscis extension (Page et al., 1998; Pankiw and Page, 2000). Other factors such as age, caste, exposure to pheromones and foraging history may also affect these thresholds (Pankiw and Page, 1999; Pankiw and Page, 2001; Pankiw and Page, 2003; Pankiw et al., 2001; Pankiw et al., 2002). Pollen foragers typically exhibit faster acquisition in associating an olfactory or tactile CS with a low concentration of sucrose (Scheiner et al., 2001). In addition, the ability of honey bees to associate a tactile CS and a sucrose US correlates to each individual's sensitivity to sucrose (Scheiner et al., 2005).

Based on these studies, we expected that, in a situation where all other variables were similar, pollen foragers would have greater sensitivity to sucrose and that they would perform better in all three appetitive learning protocols than nectar foragers. Indeed, when our subjects experienced a compound US (APC), we observed that pollen foragers achieved a higher asymptotic learning level than nectar foragers in Experiment 1. In contrast to our expectations, however, we observed that nectar foragers achieved a higher level of acquisition than pollen foragers during the proboscis-only (PC) conditioning [we failed to find a difference for the antennal-only (AC) conditioning]. As reported in the Introduction, many variables affect a honey bee's sensitivity to sucrose, including environmental variables, age and genotype of the colony (Pankiw et al., 2001). Although we collected pollen and nectar foragers using the criteria of Page et al. (Page et al., 1998), we may have included some water foragers as well. As water foragers have very low sucrose response thresholds (Pankiw and Page, 2000), it is possible that inclusion of this group affected the average level of acquisition observed for nectar foragers.

Although nectar foragers showed higher levels of response than pollen foragers during acquisition for the PC conditioning, when we examined the responses during the first recall test trial, the pollen foragers exhibited a greater probability of responding to the CS on average than the nectar foragers for all three time points we examined. Furthermore, 24 h after conditioning, significant reductions in the level of response were observed for nectar foragers conditioned with the APC or PC protocol when they were compared to the immediate test period, whereas pollen foragers did not show such reductions in recall ability at the 24 h time point. These results suggest that, as well as having differences in sucrose sensitivity and acquisition, pollen and nectar foragers may differ in their abilities to form or consolidate long-term memories. Recently, studies on lines of honey bees selected for high or low levels of foraging for pollen have shown that these strains differ in the amount of PKA and PKC present in the central brain (Humphries et al., 2003). PKA and PKC play important roles in sensory processing and the formation of memory in honey bees (Grünbaum and Müller, 1998; Friedrich et al., 2004; Müller, 2000). PKA has been previously shown to correlate with long-term gustatory responsiveness such that honey bees sensitive to sucrose also had high levels of PKA in their brains (Scheiner et al., 2003). Differences in the levels of PKA or PKC between pollen and nectar foragers may, therefore, at least partially explain the differences in learning that we observed.

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