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Thermal learning in the honeybee, *Apis mellifera*

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SUMMARY

Honeybee foragers are exposed to thermal stimuli when collecting food outside and receiving food rewards inside the nest. In both contexts, there is an opportunity for foragers to associate warmth with food rewards. However, honeybee thermal learning is poorly understood. Using an associative learning paradigm (the proboscis extension reflex), we show that honeybees can learn to associate a nectar reward with a heated stimulus applied to the antenna to mimic natural contact with a warm flower or nectar-offering forager. Conditioning with longer inter-trial intervals (ITI) significantly improved learning acquisition. We also trained bees to discriminate between temperatures above (warm) and below (cold) ambient air temperature. Learning acquisition improved by 38% per 10°C increase in absolute stimulus intensity (difference between the rewarded temperature and unrewarded ambient air temperature). However, bees learned positive temperature (warm) significantly better than negative temperature (cold) differences, approximately twice as well for 10°C as compared with a –10°C difference. Thus, thermosensation, a sensory modality that is relatively unexplored in honeybees, could play a role in the acquisition of information from nestmates (social learning) and in foraging decisions influenced by associations between floral temperature and nectar rewards.

Key words: honeybee, discrimination learning, classical conditioning, memory, thermoreception.

INTRODUCTION

Despite their small brain size and limited number of neurons relative to the central nervous systems of many vertebrates, social insects have evolved sophisticated learning and memory capabilities and are therefore important models for animal cognition (Dukas, 2008). In particular, honeybees have emerged as a major model system for the study of insect cognition because of their rich and intricate behavioral repertoire, complex learning and memory abilities, and the relative accessibility of their central nervous system (Giurfa, 2007). Honeybees can learn to associate floral odor, color and shape with a nectar reward and store this information in long-term memory (Hammer and Menzel, 1995). Associative learning is also important within the hive. Honeybees can identify nestmates from non-nestmates (Breed and Stiller, 1992) and can discriminate among queens or workers based on genetic similarity (Breed, 1981; Getz and Smith, 1983) using learned chemical cues. Multiple studies have examined their olfactory and visual learning, revealing a wide variety of phenomena, including learning generalization, extinction, memory spacing and lateralization (Hori et al., 2006; Letzkus et al., 2008; Letzkus et al., 2006; Sandoz and Menzel, 2001; Menzel et al., 2001; Sandoz and Pham-Delègue, 2004; Smith, 1991; Giurfa et al., 1996).

However, the role of an important modality, thermosensation, in associative learning remains poorly understood, although it plays an important role in colony thermoregulation (Jones et al., 2004) and, potentially, in foraging (Stabentheiner et al., 1995). Honeybees can learn to associate thermal stimuli with a sucrose solution reward (Menzel et al., 2001) but the natural role of such learning, its characteristics and the factors regulating it remain poorly understood. In fact, insect conditioning to thermal stimuli has, to date, only been examined in depth for leaf-cutting ants, who may use their capacity for thermal learning to help locate attractive sun-exposed leaves (Kleineidam et al., 2007).

Bee foragers in the field can experience temperatures below or above ambient air temperatures when foraging for nectar inside flowers (Herrera, 1995; Kevan and Baker, 1983). In the nest, honeybee foragers returning from a good food source, such as concentrated sugar solution near the nest, can warm their bodies to higher temperatures than when returning from less concentrated or more distant sources (Stabentheiner, 2001; Stabentheiner and Hagmüller, 1991). Foragers returning from natural floral nectar and pollen sources have elevated thoracic temperatures positively correlated with colony need for these resources (Stabentheiner, 2001). Such elevated temperatures could be perceived by recruits receiving food samples (trophallaxis) from these successful foragers, because their antennae contact recruiting foragers (Tautz and Rohrseitz, 1998) and contain thermosensitive sensillae (Kovac and Schmaranzer, 1996). During trophallaxis (food exchange), nectar receivers showed proboscis temperature increases of 0.85–3.5°C (Farina and Wainselboim, 2001), increases which they should be able to perceive (Heran, 1952). Honeybee workers are therefore constantly exposed to thermal stimuli during nectar foraging and exchange.

Honeybees possess paired thermoreceptive antennae (Yokohari, 1983), and thus their thermal learning may exhibit lateralization, a phenomenon observed for olfactory and visual learning (Letzkus et al., 2006; Letzkus et al., 2008). Because side-specific thermal conditioning of the PER has not been previously tested, we sought to determine if thermal learning is lateralized as well.

The time period between learning trials (inter-trial interval, ITI) affects associative memory formation. Within limits, a longer ITI results in better long-term memory for the association between multiple types of sensory stimuli (including thermal) and a nectar reward (Menzel et al., 2001). We further analyzed the spacing effect on thermal learning and investigated the possibility of differential lateralization at different ITIs. We then determined how the

magnitude of temperature differences, both positive and negative, relative to ambient air temperature, affects memory formation. We hypothesized that larger perceived temperature differences should act as more 'salient' thermal cues. In general, intense stimuli should more readily form associative memories (Rescorla, 1988), as demonstrated for learning discrimination and odorant concentration (Bhagavan and Smith, 1997).

MATERIALS AND METHODS

General methods

We used the proboscis extension reflex (PER) learning paradigm to explore the ability of honeybees to associate a sucrose solution reward (the unconditioned stimulus, US) with temperature differences (the rewarded conditioned stimulus, CS+) applied to their antennae (Takeda, 1961; Bitterman et al., 1983). This technique exploits the natural response (proboscis extension) of a honeybee to nectar (US). Following the forward pairing of a CS+ with a US, the bee will extend her proboscis if she has learned to associate the sugar reward with a temperature difference.

We conducted our experiments at the University of California San Diego in La Jolla, CA, USA (N32°52.690′, W117°14.464′) during January–June 2007 and January–March 2008. We randomly selected and captured honeybee foragers (*Apis mellifera* Linnaeus 1758) as they exited the entrances of four colonies: three colonies for the ITI experiment and one for the temperature difference experiment. We captured, chilled (4.5 min at 0°C) and harnessed foragers into stainless steel tube stands (3.7 cm long × 15 mm wide) (Bitterman et al., 1983) (Fig. 1A). Once harnessed, bees were placed in an incubator for 30 min at 30°C to increase feeding motivation. Although this fasting period is relatively short compared with other studies (Bitterman et al., 1983), we found that a 30 min fasting period was sufficient to achieve a strong and consistent level of PER response among experimental subjects. After fasting and before conditioning, bees were evaluated for spontaneous proboscis extension to unscented water or the control stimulus. However, response levels to these stimuli were quite low (ITI experiment: 0.8% of bees; temperature difference experiment: 2.8% of bees). After fasting, we also evaluated bees for their response to the US (sucrose solution). To do so, we touched an antenna (randomly choosing the left or right antenna) with a pipette tip with 1 mol l^{-1} unscented, analytical grade sucrose solution. Only bees exhibiting proboscis extension (approximately 80% of those tested) were used in the training procedure.

All studies were conducted in a temperature-controlled room (20.3±0.7°C). Yokohari reported that honeybee antennae have thermosensitive coelocapitular sensillae that are most abundant on the most distal antennal segments (Yokohari et al., 1982, Yokohari, 1983). In all experiments, we delivered the thermal stimulus by touching only the antennal tip.

ITI experiment

To deliver the thermal stimulus, we used a custom-built probe (Fig. 1A), consisting of a 12 mm × 13 mm × 2 mm copper plate attached with thermal silver epoxy (99.8% Ag, Arctic Silver, Visalia, CA, USA) at the end of a loop of copper tubing (3.25 mm diameter) through which we circulated temperature-controlled water (Haake FE2 water circulator, Thermo Haake GmbH, Karlsruhe, Germany). The ambient-air-temperature probe was a rod attached to an identical copper plate. All copper plates were half-covered with paper tape to facilitate temperature measurement with a Raytek MX6 infrared scanner (Santa Cruz, CA, USA).

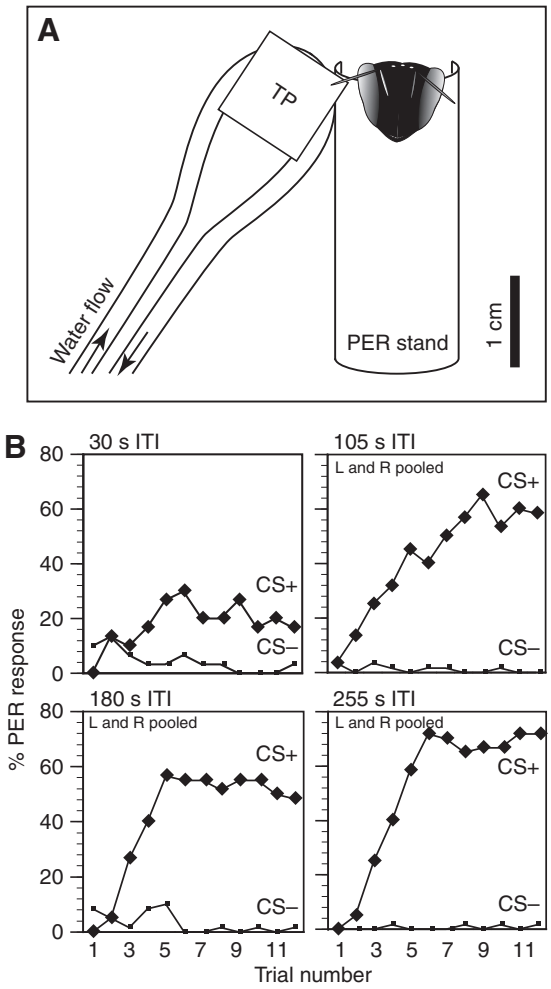

Fig. 1. (A) Schematic showing the thermal stimulus probe (TP=thermal plate attached to copper tubing through which temperature-controlled water flowed) making contact with the antenna of a bee harnessed into a proboscis extension reflex (PER) stand. (B) Effect of inter-trial interval (ITI) on learning curves. Each graph shows the percentage PER response to the rewarded conditioned stimulus (CS+) and unrewarded conditioned stimulus (CS–) in each trial for the four different ITIs used in our study (data pooled for left and right treatments because no significant lateralization effect was found).

We used a forward pairing PER design. First, each bee was randomly assigned to a side-specific treatment group. We applied the thermal stimulus only to the left antenna in the left group and only to the right antenna in the right group. We trained bees to associate a thermal stimulus with a food reward over 24 trials: 12 CS+ and 12 CS– (unrewarded conditioned stimulus) trials. A CS+ trial consisted of lightly touching the designated antenna (Fig. 1A) with the probe set at 31°C (10°C above ambient temperature) for 5 s, followed by 2 s of reward presentation (1 µl of 1 mol l^{-1} sucrose solution, equal to 30% sucrose w/w). Floral nectars occur at a variety of sugar concentrations (Baker and Baker, 1982), and generalist bee foragers collect nectars ranging from 10% to 70% sugar w/w (Roubik et al., 1995). To elicit proboscis extension, we first touched the designated antenna with a pipette bearing a droplet of sucrose solution and then provided the reward when the bee extended her proboscis. The CS– consisted of lightly touching the room-temperature probe to the designated antenna for 5 s without a subsequent sucrose reward. These trials were pseudorandomly

alternated over the course of the experiment in the pattern AABABBABAABABBABAABABBAB with A being a conditioned trial (CS+) and B being an unconditioned trial (CS–) (Bitterman et al., 1983).

Touching the antenna provided visual and mechanical stimuli in addition to a thermal stimulus. However, the visual and mechanical stimuli were identical in CS+ and CS– trials, and thus a bee responding preferentially to the CS+ (Fig. 1B) did so because she had conditioned to the thermal stimulus.

We scored a bee's response during the 5 s of exposure to the CS, before the US was presented (1=extension of proboscis past mandibles, 0=no extension of the proboscis past mandibles). For each bee, we used one of four ITIs (30 s, 105 s, 180 s or 255 s). We chose the 30 s and 180 s intervals because they were used in a previous experiment testing honeybee thermal learning (Menzel et al., 2001). The remaining two ITIs were intermediate values chosen to evaluate fine-scale differences in learning performance. Thus, we chose four different ITIs [as compared with two (Menzel et al., 2001)] to better evaluate the influence of ITI on learning acquisition in our analysis model. We tested 60 bees (30 left group and 30 right group) at each ITI, for a total of 240 bees (80 bees from each colony). After trials with each bee, we thoroughly cleaned the probe plates with 100% ethanol.

Temperature difference experiment

We captured, harnessed and incubated bees before conditioning as in the previous experiment. To generate temperatures above and below ambient air temperature, we used a Peltier chip (model ET1.5-18-F2A-H4-C1, Melcor Thermoelectric Cooler, Trenton, NJ, USA) with a metalized ceramic surface that can heat or cool to a set temperature (2–31.5°C), depending upon the voltage polarity and current applied. We attached the chip to a copper tube probe as previously described and stabilized chip temperature by circulating heated or chilled water as appropriate (Fig. 1A). We built two Peltier probes, of which one served as the room-temperature probe and was not heated or cooled. We monitored probe temperatures and cleaned them as in the ITI experiment.

In the ITI experiment, learning acquisition was best with an ITI of 255 s and reached a plateau after 5–6 trials (Fig. 2). We therefore used an ITI of 255 s in the temperature difference experiment, and reduced the number of trials to 20 (10 CS+ and 10 CS– trials in the order ABBABAABABBABAABABBA). We used a 1 mol l^{-1} sucrose solution as the reward (US). Approximately 20 bees per temperature treatment were used. In some cases, we tested differences smaller than 0.25°C (Fig. 3B). Thus, in order to ensure that each bee was adequately exposed to each temperature stimulus, we touched both antennae for all tested temperature differences. Left (L) bees were touched on their left antenna first, then their right antenna second. Right (R) bees were touched on their right antenna first, then their left antenna second. Each antennal contact lasted for 2.5 s (total 5 s exposure). In CS+ trials, the experimenter touched the pipette tip with a droplet of sucrose solution to both antennae (in the same order as the thermal stimulus) to elicit proboscis extension and then rewarded the bee. For example, L bees were touched first on their left and then on their right antenna before the reward was given.

Statistical analysis

For all tests, we used JMP IN v4.04 software (SAS®, Cary, NC, USA). We analyzed learning acquisition with a discrimination index (DI), the sum of a honeybee's responses to the CS– subtracted from the sum of her responses to the CS+ (Pelz et al., 1997). In the temperature difference experiment, if a forager learns after only one trial (maximum possible learning), her DI will be 9 because she responds with PER to all CS+ after the first conditioning trial and to no CS–. To evaluate learning performance at the end of training, we also measured the ability of bees to discriminate the thermal stimulus from the control for the final stimulus trial. This index,

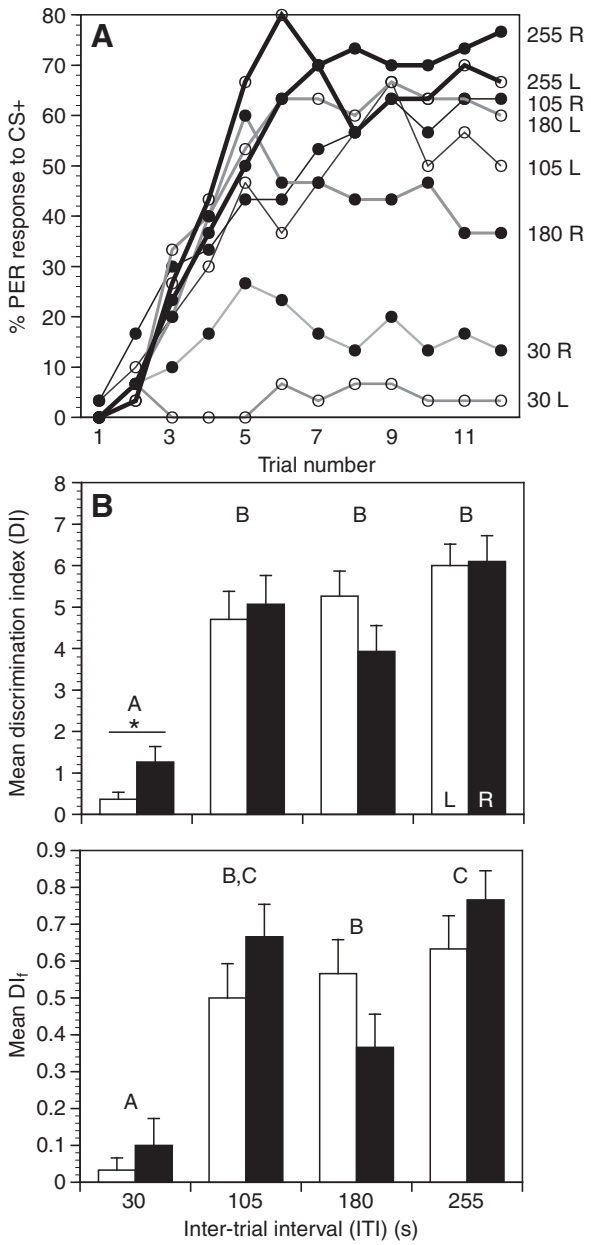

Fig. 2. The effect of inter-trial interval (ITI) on thermal learning. (A) Learning curves for the total conditioned stimulus (CS+) proboscis extension reflex (PER) response over all rewarded trials are shown, divided into left (L, open circle) or right (R, filled circle) antennal treatments. The ITI corresponding to each learning curve is shown on the right, next to the results of the last trial. (B) The mean discrimination index (DI) for the different ITIs is shown, divided into left (L, open bars) and right (R, filled bars) treatments. Error bars show standard errors. Significant differences between ITI treatments (based upon Tukey HSD tests) are indicated with different letters. In this experiment, the maximum DI is 11 (the bee responds with PER to all CS+ after the first conditioning trial and no CS–) and the maximum final discrimination index (DI_f) is 1.

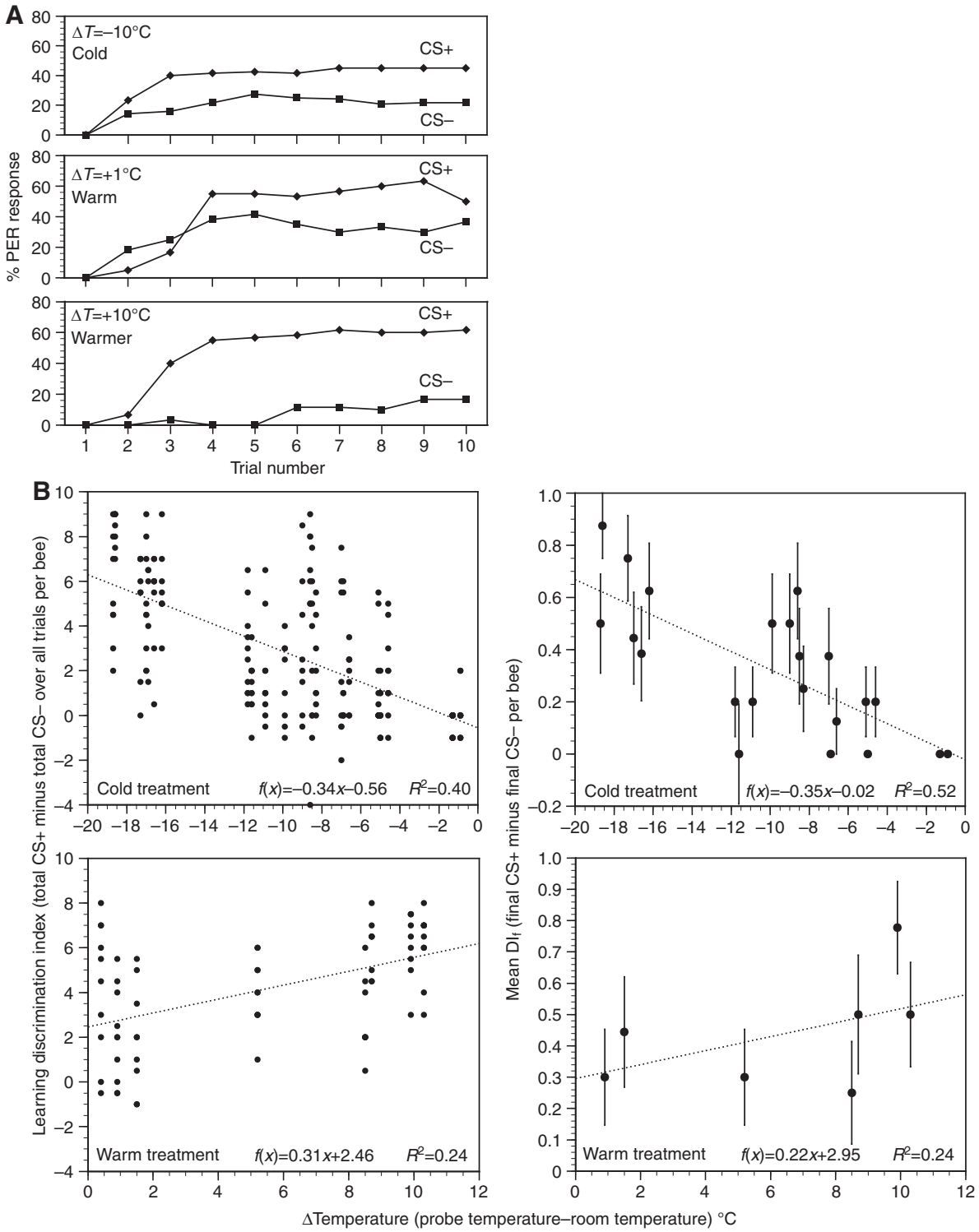

Fig. 3. The effect of temperature (*T*) (cold *vs* warm treatments) on thermal learning. (A) Learning curves at three temperature differences (–10°C, 1°C and 10°C) over all 10 learning trials. The total conditioned stimulus (CS+) (filled diamonds) and CS– (filled squares) proboscis extension reflex (PER) responses per trial are shown (30 bees per temperature treatment). (B) Learning discrimination index (DI) values per bee (the total number of conditioned PER responses minus the total number of unconditioned PER responses per bee) are shown for the cold and warm treatments in separate plots. We also show the mean final discrimination index (DI_f) value with standard error for each temperature difference, separated into cold and warm treatment plots. In this experiment, the maximum DI value is 9 (the bee responds with PER to all CS+ after the first conditioning trial and no CS–). The maximum DI_f value is 1. Dashed linear regression lines with corresponding regression equations and R^2 values are shown.

DI_f, can be a more sensitive parameter for assaying learning and was calculated by subtracting the final CS– response from the final CS+ response. All data met assumptions of normality, and thus we performed Student's *t*-tests and analysis of variance (ANOVA). In the ITI experiment, we tested colony as a random effect (Standard Least Squares using an EMS algorithm) and ITI and lateralization

(left or right treatment) as fixed effects. In the temperature difference experiments, we tested absolute temperature difference, treatment (cold or warm) and lateralization (left or right first antennal stimulation) as fixed effects. We included all appropriate fixed-effect interactions in our full models.

We used *t*-tests to evaluate the hypothesis that bees can discriminate between the rewarded thermal stimulus and the unrewarded control stimulus (mean DI is greater than zero). We performed paired *t*-tests to determine if responses to CS+ were significantly greater than responses to CS– for the same individual. For comparisons of variance, we used Levene's test for equality of variances. Where appropriate, we applied a Sequential Bonferroni correction using the Dunn–Sidak method to correct for type I error (Sokal and Rohlf, 1995) and note if a result is significant (*) or not (NS) after this correction. Pairwise comparisons were performed with *post-hoc* Tukey–Kramer Honestly Significant Difference (HSD) tests.

RESULTS

ITI experiment

Honeybees learned to associate a temperature difference with a food reward, and learning acquisition increased with reinforcement (Fig. 1B, Fig. 2). Out of 240 honeybees used, over 70% displayed at least one response to the CS+ after conditioning trials, and responses increased over the course of training (Fig. 1B). The DI and DI_f indices were significantly greater than zero for all ITI greater than 30 s (Table 1). For DI, but not DI_f, the 30 s right antennal treatment group also showed significant learning (Table 1). Thus, bees successfully distinguished between the heated probe (CS+) and the room-temperature probe (CS–) for ITI ≥30 s.

Analysis of DI (learning summed over all trials) revealed no significant interaction between ITI and lateralization ($F_{1,235}$=1.2, P=0.28), and thus we ran a simplified three-factor model. In this model, there was no significant effect of colony ($F_{1,236}$=2.4, P=0.12) or lateralization ($F_{1,236}$=0.0004, P=0.98). However, there was a highly significant effect of ITI ($F_{1,236}$=42.0, P<0.0001), and ITI accounted for 20% of variation in DI. Learning was significantly poorer in bees trained with a 30 s ITI as compared with all other intervals (*post-hoc* Tukey HSD, Q=2.59, P<0.05) (Fig. 2B) but there were no significant differences in learning among 105 s, 180 s and 255 s intervals. There were no significant learning differences between antennal treatment groups within each ITI treatment (2-tailed t_{41}≤2.21, P≥0.033, NS after Sequential Bonferroni correction) (Fig. 2A).

We also evaluated discriminating learning at the ending of training (DI_f). The results matched our DI analyses. There was a highly significant effect of ITI ($F_{3,232}$=22.7, P<0.0001*) such that DI_f increased with increasing ITI, and ITI accounted for 29% of variation in DI_f. The ITIs of 30 s, 180 s and 255 s were all significantly different (Tukey HSD, Q=2.58, P<0.05) (Fig. 2B). There was no significant interaction of ITI and lateralization ($F_{3,232}$=2.07, P=0.10), and no significant effect of lateralization ($F_{1,232}$=0.33, P=0.56). There were no significant learning differences between antennal treatment groups within each ITI treatment (2-tailed t_{41}≤0.83, P≥0.41) (Fig. 2A).

Temperature difference experiment

Bees learned to associate a wide range of temperature differences with a food reward, including temperatures below and above ambient air temperatures (20.3±0.7°C). In general, learning responses to the CS+ reached a plateau after the fourth reinforcement trial (Fig. 3A). Learning occurred even for a CS+ that was only 0.4°C above ambient air temperature (DI_f analysis, 2-tailed paired *t*-test, t_9=4.58, P=0.001*) (Fig. 3A).

Using the DI, we tested the effect of absolute temperature difference (the absolute magnitude of the temperature difference), treatment (cold or warm) and lateralization (left-first or right-first antennal stimulation). Interactions were not significant (interaction effect tests: $F_{1,274}$≤3.70, P≥0.06). In our three-factor model, absolute temperature difference ($F_{1,278}$=163.7, P<0.0001*) and treatment ($F_{1,278}$=72.7, P<0.0001*) were the only significant effects. There is no significant effect of lateralization ($F_{1,278}$=0.22, P=0.64).

The DI_f analysis yielded the same results. No interactions were significant ($F_{1,274}$≤3.29, P≥0.07). In the three-factor model, absolute temperature difference ($F_{1,278}$=28.4, P<0.0001*) and treatment ($F_{1,278}$=18.8, P<0.0001*) were significant but lateralization ($F_{1,278}$=0.6, P=0.64) was not.

Bees showed higher learning acquisition for larger absolute temperature differences between CS+ and ambient air temperatures (Fig. 3B, absolute temperature difference effect). Thus, bees were able to associate sucrose solution rewards with cold or warm temperature differences. The absolute magnitudes of the learning slopes were similar [no significant interaction of treatment and absolute temperature difference (DI: $F_{1,274}$=3.70, P=0.06; DI_f: $F_{1,274}$=2.23, P=0.14)]. In the cold treatments, the DI increased by 3.4 PER responses per 10°C increase in absolute temperature difference (mean DI_f increased by 3.5 PER responses per 10°C increase). Thus, there was a 38% increase in learning acquisition (relative to the maximum possible learning, DI=9) with each 10°C increase in absolute temperature difference. In the warm treatments, learning DI and mean DI_f increased at a similar rate: 3.1 and 2.2

Table 1. Results of one-tailed *t*-tests to determine if associative learning occurred [mean discrimination index (DI) is significantly greater than zero] at different inter-trial intervals (ITI)

ITI (s)	Antenna stimulated	Mean DI±s.e.	DI t_{29}	DI P	Mean DI_f±s.e.	DI_f t_{29}	DI_f P
30	Left	0.37±0.17	2.16	0.04 (NS)	0.03±0.03	1.00	0.16
30	Right	1.23±0.37	3.41	0.0019*	0.10±0.07	1.36	0.09
105	Left	4.70±0.67	6.96	<0.0001*	0.50±0.09	5.39	<0.0001*
105	Right	5.07±0.69	7.31	<0.0001*	0.67±0.09	7.62	<0.0001*
180	Left	5.27±0.60	8.80	<0.0001*	0.57±0.09	6.16	<0.0001*
180	Right	3.93±0.62	6.32	<0.0001*	0.37±0.09	4.10	<0.0001*
255	Left	6.00±0.52	11.52	<0.0001*	0.63±0.49	7.08	<0.0001*
255	Right	6.10±0.62	9.78	<0.0001*	0.77±0.08	9.76	<0.0001*

At all ITIs, regardless of which antenna is stimulated, the DI is significantly greater than zero (one-tailed *t*-test), with the exception of the left antennal treatment at 30 s (not significant, NS, after Sequential Bonferroni correction). For the final discrimination index, DI_f, left and right antennal treatments at 30 s do not result in significant learning, although there is significant learning at all longer ITI. The mean DI and corresponding standard error (s.e.) is given for each treatment group (N=30 bees per group). All P-values marked with an asterisk are significant after Sequential Bonferroni correction.

PER responses per 10°C, respectively (34% increase in DI learning relative to maximum possible learning).

There is a significant difference in learning acquisition for cold *vs* warm treatments that is shown in the *y*-intercepts of the learning discrimination regression lines (DI: –0.56 for cold and 2.45 for warm treatments; mean DI_f: –0.02 for cold and 0.30 for warm treatments). Thus, for any given temperature difference, the absolute value of the learning DI is greater for a warm (positive) as compared with a cold (negative) temperature difference. This is illustrated in Fig. 3A, which shows learning acquisition curves for a –10°C and a +10°C conditioning stimulus. Based upon regression equations, the estimated DI is 2.84 PER responses for a –10°C treatment and 5.56 PER responses (96% more) for a 10°C treatment. The estimated mean DI_f is 0.32 PER responses for a –10°C treatment and 0.52 PER responses (60% more) for a 10°C treatment.

There was no significant variation in learning performance between bees trained to the greatest warm temperature difference (+10°C difference) and those trained to the largest cold temperature difference (–19°C difference, Levene's test: DI_f, $F_{1,33}$=0.46, P=0.50; DI, $F_{1,33}$=4.78, P=0.04, NS after Sequential Bonferroni Correction).

DISCUSSION

These experiments provide the first demonstration that thermal learning acquisition increases as the absolute temperature difference between the CS+ and CS– increases, regardless of whether these differences are positive or negative. In warm treatments, there is a 34% increase in DI learning acquisition for each 10°C increase in temperature difference, relative to maximum possible learning. Bees also learned to associate a lower-than-ambient temperature with food reward (cold treatments), although such learning was not as strong as for warm treatments (Fig. 3). These effects were pronounced and consistent whether measured using a total DI (difference between all CS+ and CS– responses summed over all trials per bee) or a DI_f (the final learning performance calculated as the difference between the final CS+ response and the final CS– response).

Our results demonstrating that a longer ITI improves memory acquisition, complement the findings of Menzel et al. (Menzel et al., 2001) on the conditioning of thermal stimuli and conditioning in other sensory modalities. Interestingly, we did not find strong evidence for the Kamin effect, the decay in retrieval performance due to the interference of new and previously acquired information (Kamin, 1957). For olfactory learning, the Kamin effect is shown by a learning performance 'dip' at an ITI of 3 min (Gerber and Menzel, 2000). In our ITI experiment, there is no significant decrease for the 3 min (180 s) ITI as measured by the DI. However, the DI_f (which measures final learning performance and may thus be a more sensitive measure) shows that learning is reduced at the 180 s ITI relative to the 255 s ITI (Fig. 2B).

If one considers the mean responses pooled for left and right antennae (because there is no lateralization effect), at a 180 s ITI there is 20% DI_f decrease (NS) in learning relative to the shorter 105 s ITI. There is also a 33% DI_f decrease (significant) in learning relative to the longer 255 s ITI. The magnitude of the Kamin effect may be smaller or there may be greater individual variation in thermal learning as compared with olfactory learning. Thus, results from olfactory learning may not be completely transferable to the thermal modality, and the latter may exhibit different memory dynamics. To directly compare thermal PER data to olfactory PER studies, it will be necessary to use ITI treatments at 1 and 10 min with identical bee handling procedures and test for long-term memory retention (Gerber and Menzel, 2000).

Side-specificity (lateralization) in honeybee thermal learning had not been investigated. We found no significant side-biased learning when both antennae are stimulated with thermal and sucrose stimuli (both antennae tapped with sucrose to elicit proboscis extension, temperature difference experiment). Likewise, there is no significant lateralization effect when bees receive thermal stimuli on only one antenna (left or right, ITI experiment). At the lowest level of learning acquisition (30 s ITI) in this experiment, there is no significant learning when only the left antenna is conditioned, although there is when only the right antenna is conditioned (based upon DI) (Table 1). Nonetheless, the distributions of left and right treatment groups (DI, P=0.033, NS) are not significantly different after Sequential Bonferroni correction. There are no significant differences between left and right at any ITI for DI_f. Thus, there is no compelling evidence for lateralization based upon our results and the methods that we used.

Some factors that have been shown to affect honeybee PER responses, such as genotype, feeding status and foraging role (Page et al., 1998), were not tested in the present study, in which bees were collected randomly as they exited the hive entrance. This sampling method could introduce variance into our results. Future thermal learning studies could examine how factors such as genotype, forager age and foraging specialization influence thermal conditioning. In addition, some variance could have been introduced by our relatively short fasting period of 30 min. However, 80% of foragers responded to the sucrose solution after this fasting period, and those that responded learned rapidly and achieved plateau learning levels similar to that observed in other PER experiments with longer fasting periods (Menzel et al., 2001).

Temperature difference effect

Learning acquisition is correlated with the absolute temperature difference (Fig. 3). Honeybees may be able to detect larger temperature differences more easily than smaller temperature differences (Heran, 1952). Larger temperature differences should generate a more intense stimulus that could promote associative memory formation (Rescorla and Wagner, 1972). For example, olfactory discrimination learning increases with higher odorant concentrations (Bhagavan and Smith, 1997). With respect to the cold treatment CS+, there is a potential aversive effect of cold temperature (such as decreased appetitive motivation) that may have led to reduced PER learning (Menzel and Müller, 1996). A slight cold amnesia effect (decreased neural activity affecting memory formation and retrieval) in addition to decreased motor performance is also possible, although we contacted only the tips of each bee's antenna for a brief period (total of 5 s per trial). Such effects could contribute to bees learning warm temperature differences 60% (DI_f) to 90% (DI) better then cold temperature difference of comparable magnitude (calculated for stimuli 10°C above as compared with 10°C below ambient air temperature) (Fig. 3).

Natural context

There are two main natural contexts for honeybee thermal learning: nectar rewards outside the nest and nectar exchange inside the nest. Outside the nest, floral warmth can provide an energetic reward to pollinators (Seymour et al., 2003; Dyer et al., 2006; Rands and Whitney, 2008). Bees can experience warmer than ambient air temperatures when collecting floral nectar rewards (up to 8°C higher inside than outside *Narcissus longispathus* (Amryllidaceae) flowers), and while foraging in the sun as compared with the shade (Kevan and Baker, 1983). Honeybee foragers may also be able to associate floral temperatures with nectar rewards.

In bumblebees, Whitney et al. showed that floral warmth could function as a cue independent of a sucrose reward and suggested that pollinators could benefit from increased nectar and pollen rewards if they can identify warm flowers (Whitney et al., 2008). Interestingly, they demonstrated that bumblebees could use lower temperatures as a cue to identify higher sucrose rewards, a situation analogous to our conditioning honeybees to associate cooler temperatures with sucrose reward.

A honeybee's ability to associate positive temperature differences with nectar rewards could also have a natural role inside the nest. Honeybee foragers can elevate their body temperature after returning from a high-quality artificial food source (Stabentheiner and Hagmüller, 1991), and foragers returning from natural nectar or pollen sources increase their thoracic temperature when the colony has need for these resources (Stabentheiner, 2001). Foragers returning from natural food sources had thoracic temperatures ranging from 31.4 to 43.0°C (nectar foragers) and 31.7°C to 41.4°C (pollen foragers) while ambient air temperatures on the dance floor were 26.0–36.0°C (during nectar foraging) and 27.2–36.0°C (during pollen foraging) (Stabentheiner et al., 2001). These temperature differences are consistent with the range of temperatures used in our warm treatment (Fig. 3). Foragers can also be warmed during trophallactic food-transfers (receiver proboscis temperature increases by 0.85–3.5°C) (Farina and Wainselboim, 2001). In addition, dance followers make antennal contact with waggle dancing bees (Tautz and Rohrseitz, 1998; Stabentheiner et al., 1995) that periodically stop and provide brief nectar samples as a part of recruitment (von Frisch, 1967).

Thus, bees experience a wide range of temperatures while foraging for nectar in the field and while receiving nectar inside the nest. Our results show that honeybees can learn to associate nectar rewards with temperature differences above or below ambient environmental temperatures and suggest that thermosensation, a sensory modality that is relatively unexplored in honeybees, could play a role in the acquisition of information from nestmates (social learning) and in foraging efficiency.

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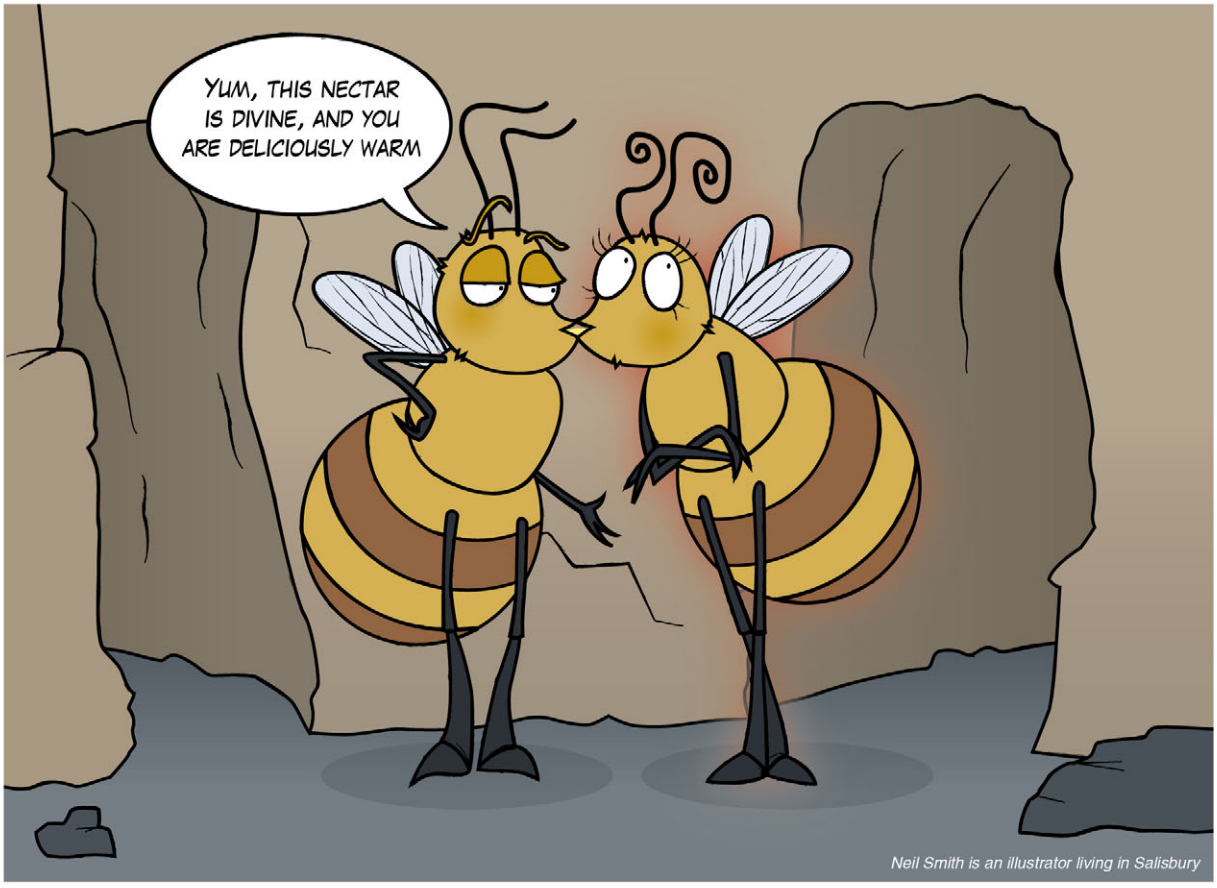
BEES DISCRIMINATE BETWEEN HOT AND COLD FOOD



Getting a temperature is a bad thing for most mammals, but returning forager bees always warm up on the home run and a high temperature can indicate the quality of nectar or pollen that they return with. Flowers also warm up: the temperature inside a *Narcissus longispathus* can be 8°C higher than the surroundings. Knowing that bees sense temperatures with their antennae, James Nieh, and his colleagues Tobin Hammer and Curtis Hata from The University of California San Diego, wondered whether bees can use this thermal information about food. Can bees learn to discriminate between food sources at different temperatures (p. 3928)?

Training bees to stick out their tongues in return for a sugary reward when the team touched a warm surface to a bee's antenna, the team discovered that bees can learn to identify warmth with food. Next the trio tested whether the insects could learn to associate temperature differences with a food reward, and discovered that the bees do associate temperature differences with food. The bees' ability to recognise the temperature difference increased dramatically as the difference increased, but the insects were better at recognising warm temperature differences than cold temperature differences.

So, bees can learn to recognise different temperatures and could be guided by this information when foraging for, or receiving, food.

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