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Floral tea polyphenols can improve honey bee memory retention and olfactory sensitivity

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ABSTRACT

Animal-pollinated plants face a common problem, how their defensive anti-herbivore compounds may impair or alter pollinator behavior. Evolution has tailored multiple solutions, which largely involve pollinator tolerance or manipulation, to the benefit of the plant, not the removal of these compounds from pollen or nectar. The tea plant, *Camilla sinensis*, is famous for the caffeine and tea polyphenols (TP) that it produces in its leaves. However, these compounds are also found in its nectar, which honey bees readily collect. We examined the effects of these compounds on bee foraging choices, learning, memory, and olfactory sensitivity. Foragers preferred a sucrose feeder with 100 μg or 10 μg TP/ml over a control feeder. Caffeine, but not TP, weakly increased honey bee learning. Both caffeine and TP significantly increased memory retention, even when tested 7 d after the last learning trial. In addition, TP generally elevated EAG responsiveness to alarm pheromone odors. These results demonstrate that other secondary plant compounds, not only caffeine, can attract pollinators and influence their learning and memory.

1. Introduction

Multiple plant species produce defensive compounds that deter herbivory (Sullivan et al., 2008, Harborne, 1993). Such chemicals are also consumed by pollinators, but there has evidently been little selective pressure for plants to exclude these compounds from nectar and pollen (Gegear et al., 2007; Irwin et al., 2014; Stevenson et al., 2017; Jacobsen and Raguso, 2018; Jones and Agrawal, 2016). In fact, plants can benefit from such compounds if they increase pollinator specialization, reduce nutrient degradation in nectar, decrease pollinator diseases, or reduce nectar robbing (Stevenson et al., 2017). Through coevolution (Jacobsen and Raguso, 2018), pollinators have also adapted to these compounds (Jones and Agrawal, 2016). For example, the Asian honey bee, *Apis cerana*, does not prefer to forage on the toxic, triptolide-containing nectar of the thunder god vine, but will do so at times of relative floral dearth and suffer relatively mild effects: decreased olfactory memory after an acute exposure, but no learning or memory effects after chronic exposure (Zhang et al., 2018).

Caffeine, common in *Coffea* and *Citrus* species, may increase plant fitness by enhancing honey bee olfactory cognition (Wright et al., 2013, Sharma et al., 1986) through improving learning in *Apis mellifera* (Couvillon et al., 2015; Mustard et al., 2012; Si et al., 2005; Wright et al., 2013). Wright et al. (2013) reported a range of natural caffeine levels (0.003 to 0.253 mM) and showed that acute doses of 0.1 mM caffeine and higher enhanced memory. A low caffeine concentration in nectar can increase pollinator visitation (Singaravelan et al., 2005). However, such cognitive effects have not been documented for other secondary compounds, and we therefore sought to test if tea polyphenols, another group of secondary compounds likely produced for plant defense and found in tea nectar (Sharma et al., 1986), have similar benefits for plants: attracting bee pollinators and enhancing their olfactory memory.

In China, bees are potentially exposed to caffeine and TP in the nectar of tea (*Camilla sinensis*), a widely cultivated crop (Sharma et al., 1986). *Camilla sinensis* flowers from August to February, a time of

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relative floral dearth. Although *C. sinensis* co-evolved with Asian honey bee species such as *A. cerana*, the introduced European species, *A. mellifera*, is now widespread in China where it is used for pollination and honey production (Yang, 2005). We therefore tested if TP can alter *A. mellifera* foraging preferences and if TP and caffeine can alter *A. mellifera* learning and memory and antennal responsiveness to odors, measured via electroantennograms (EAG). Honey bees will avoid inflorescences at which they detect alarm pheromones, signs of past danger (Wen et al., 2017a; 2017b). Such avoidance of dangerous inflorescences can decrease plant fitness (Romero et al., 2011). If TP increases bee sensitivity to bee alarm odors, an interesting side effect could arise, with plants suffering potentially decreased pollination but bees increasing their fitness via enhanced danger avoidance. We therefore tested if TP could increase honey bee antennal responsiveness to alarm pheromone components.

2. Materials and methods

2.1. Colonies and sites

We used three (Exp 3 and Exp 4) or four (Exp 2) *Apis mellifera*colonies maintained at the apiaries of the Eastern Bee Institute of Yunnan Agricultural University, Yunnan, China (GPS coordinates: 25.128849 N, 102.752200E). Experiments were conducted from August 2018 to February 2019. Colonies were in good condition, based upon standard inspection methods (Vincent et al., 2013) and engaged in natural foraging. Samples sizes are given in the figure legends and in Tables S1 and S2.

2.2. Experiment 1. Caffeine and TP natural percentage within the tea nectar

2.2.1. Sample collection

We collected *Camilla sinensis* tea nectar from Yunnan Agricultural University during its flowering season from November to December in 2018. We collected tea nectar from 8:00 a.m. to 10:00 a.m. with a microsyringe (10 ul, Shanghai Anting Co., Ltd. China) and obtained a total of >10 ml (10 tubes, 1 ml per tube, corresponding to the nectar contents of >100 flowers per tube), which was immediately stored at 4 °C at the end of each collection day.

2.2.2. Concentrations of caffeine and TP in tea nectar

We used an Agilent 1200-UV variable wavelength detector (at 280 nm) to measure caffeine and TP concentrations in natural tea nectar with HPLC (Zhou et al., 2013) and a TSK-GEL ODS-80TM (4.6 mmi × 250 nm) column using a semi-quantitative method. Mobile phase A consisted of CH_3CN (5% v/v) in a H_3PO_4(0.261% v/v) solution. Mobile phase B was CH_3ON (40% v/v) in a H_3PO_4 (0.261% v/v) solution. Elution gradient separation was performed as follows: 0–20 min with 10% mobile phase B and 90% mobile phase A; 20–20.1 min with 22% B and 78% A; 20.1–26 min with 100% B and 0% A; 26–26.5 min with 100% B and 0% A; 26.5–27 min with 10% B and 90% A; and finally held for an additional 5 min. The flow rate was 1 ml/min, and the injection volume was 2.0 ul for each analysis. We conducted 10 technical replicates: 10 different samples in 10 different runs (total of 20 μl of nectar). Standards were purchased (DASF Biology Co., Ltd. Nanjing, China, Fig. 1).

C. sinensis polyphenols in can differ according to the plant part analyzed and consist of a mixture of several compounds including gallic acid (GA), epigallocatechin (EGC), catechin (C), epicatechin (EC),

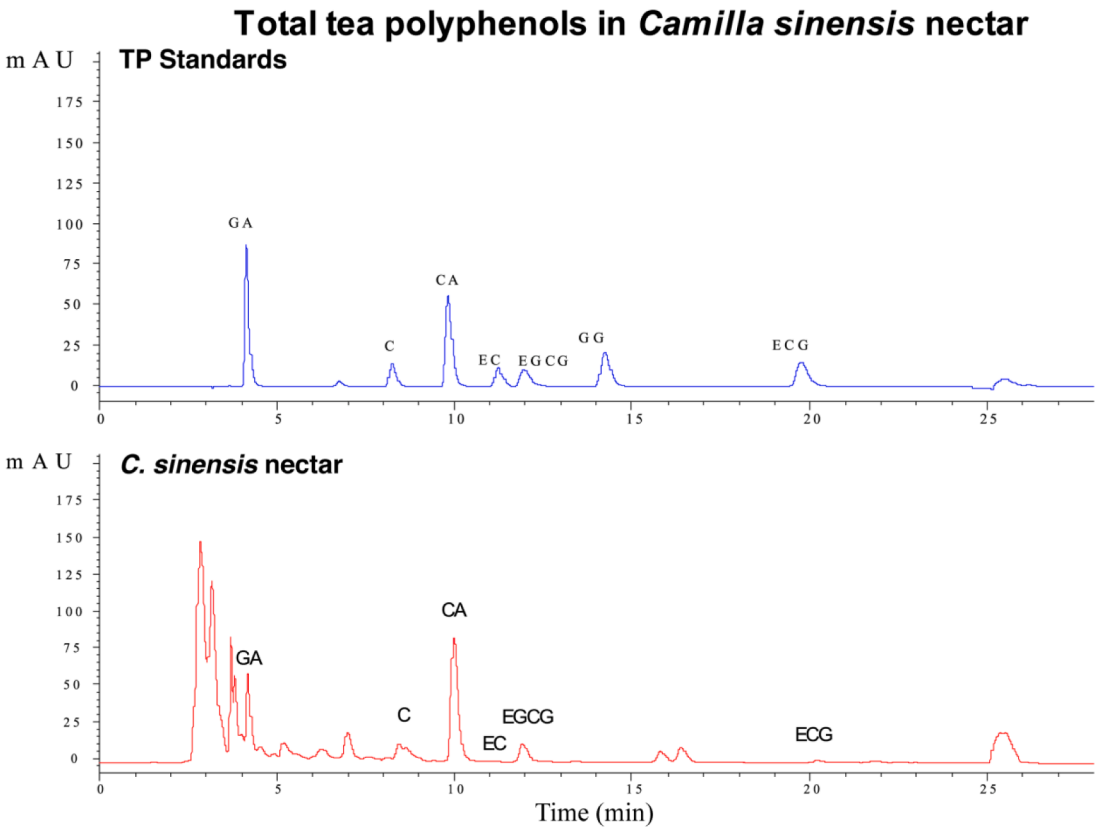

Fig. 1. Chromatograms showing the relative abundance of caffeine (CA) and total tea polyphenol compounds in *C. sinensis* nectar with reference to TP standards. Abbreviations represent gallic acid (GA), epigallocatechin (EGC), catechin (C), caffeine (CA), epicatechin (EC), epigallocatechin gallate (EGCG), 1,4,6-tri-O-galloyl-β-D-glucose (GG), and epicatechin gallate (ECG).

epigallocatechin gallate (EGCG), and 1,4,6-tri-O-galloyl-β-D-glucose (GG), and epicatechin gallate (ECG) (Morikawa et al., 2013; Lin et al., 2003). Based upon the TP concentrations measured in natural *C. sinensis* nectar, we created a synthetic TP solution containing the same relative proportions of each TP compound (GA, SG8050; EC, SE8100; EGCG, E8120; ECG, IE0130. Solarbio, ≥98.0% purity, China), with the exception of caffeine. We created 10 and 100 ug/ml TP solutions to bracket the naturally occurring concentrations of TP (see Table 1). These synthetic TP solutions did not contain EGC (because we did not detect EGC levels in natural tea nectar) or caffeine (since we wished to test the effects of TP compounds separately from caffeine).

2.3. Experiment 2. Choice preference test

We bioassayed TP nectar preferences with four colonies of *A. mellifera*. We trained bees to a grooved plate feeder (5.0 cm diameter and 6.5 cm high) with a circle of green paper placed underneath to facilitate visual orientation. We trained bees by placing the feeder on a plastic stool 100 m from the focal colony, capturing departing foragers from the focal colony with a 20 ml glass vial, releasing them at the feeder, and marking bees that fed with a numbered bee tag (Opalith-Zeichenplättchen) affixed to the thorax with shellac. We repeated this training procedure until 20 bees from the focal colony reliably and repeatedly visited the feeder. An observer at the focal colony verified the return of our numbered bees. All unmarked bees from focal or other colonies were captured with aspirators. We trained on one day and tested on the subsequent day. Once our marked bees began foraging again at the training location, we captured all but one forager with an aspirator to ensure that each bee made an individual choice in the absence of other bees. This holding aspirator was kept in the shade to keep the bees in good condition. We then waited for the focal forager to leave the feeder, cleaned the stool with 100% ethanol, and set out two identical clean feeders 20 cm apart at the same location. After analyzing natural tea nectar, we measured an average of 19.1 ± 0.56 μg/ml of TP compounds (excluding caffeine, Table 1). In our choice bioassay, we therefore chose to test three different total TP concentrations: 0 μg/ml (control), 10 μg/ml (low TP),and 100 μg/ml (high TP, not field-realistic).The feeders offered the following paired choices (all in 30% sucrose solution w/w): 0 vs. 10 μg/ml TP, 0 vs 100 μg/ml TP, or 10 vs 100 μg/ml TP. We tested 20 bees per choice type per colony and used four different colonies (total of 240 bees).

Once the focal forager returned, it would often sample both feeders, but we only scored a choice if it fed >10 s on one feeder. Between each trip, we set out clean feeders and swapped their positions to avoid site biases. We assayed the choice of each focal bee over 10 trips to the feeder array and then removed it with a separate aspirator. We then cleaned the stool again and replaced the array with a clean set of feeders, released a marked bee from the holding aspirator, and used it as the next focal bee.

During the trial, we continued to remove all other bees, only counted choices made in the absence of all other bees at the feeder, rotated the feeders 180° after each choice to exclude potential side bias, and replaced the feeders with clean ones after each choice to remove olfactory cues. The feeder monitor sat directly behind and between the feeders, allowing bees to fly unimpeded from the nest to the array.

Table 1
The concentration of caffeine and tea polyphenols in nectar collected from *C. sinensis* inflorescences and in the TP solutions fed to bees (mean ± 95% CI). We tested for the presence of EGC in tea nectar because this compound has previously been reported in TP extract from other parts of the plant. However, we detected no EGC in tea nectar. The TP synthetic solution fed to bees contained no caffeine because we wished to separately test the effects of TP apart from caffeine.

	Component	Concentration (μg/ml)	Molarity (mM)
C. sinensis nectar	Caffeine (CA)	15.83 ± 0.06	0.0792
	Gallocatechin (GA)	7.87 ± 0.38	0.0257
	Epicatechin (EC)	1.13 ± 0.07	0.0039
	Epigallocatechin gallate (EGCG)	9.18 ± 0.10	0.02
	Epicatechin gallate (ECG)	0.921 ± 0.01	0.0021
	Epigallocatechin (EGC)	0	0
TP solution fed to bees	Gallocatechin (GA)	7.82	0.0255
	Epicatechin (EC)	1.17	0.004
	Epigallocatechin gallate (EGCG)	9.24	0.0201
	Epicatechin gallate (ECG)	0.94	0.0021
	Epigallocatechin (EGC)	0	0

2.4. Experiment 3. Learning and memory in honey bees

2.4.1. Sample collection

We used aspirators to collect returning foragers from the entrances of three colonies between 9:30 a.m. to 11:30 a.m. on sunny days (sample sizes in Table S1). We individually fed each bee with 15 μl of 30% (w/w) pure sucrose solution with a micropipette and then caged them (no more than 100 individuals with one colony per cage) in wood cages (20 cm × 20 cm × 12 cm) in an incubator overnight (25 °C, 65% relative humidity). Following standard protocols (Giurfa and Sandoz, 2012), all bees were starved overnight to facilitate successful conditioning.

2.4.2. Classical olfactory conditioning

To prepare bees for PER, we placed each bee in a clean glass vial on ice for approximately 5 min until movement significantly diminished. To restrain the bees for PER, we placed them in 0.5 ml plastic centrifuge tubes that had holes cut from their tips, allowing only the bee heads, mouthparts, and antennae to emerge (Gong et al., 2016). Bees were able to move their heads and proboscises and were trained 5 h later. Olfactory learning and memory were tested with a PER conditioning assay (Bitterman et al., 1983). During each trial, bees were exposed to a continuous air flow of 0.5 L min^{-1} through a syringe (60 ml, inner diameter of 3 mm). The olfactory conditioned stimulus (CS) was 5 μl of hexane (Sigma-Aldrich, St Louis, MO, USA) dispensed onto a filter paper (1 cm × 1 cm) inside a syringe (Gong et al., 2018). Hexane is typically not used as a conditioning odor for honey bees because it lacks the salience of some other odors (Wright and Smith, 2004). However, preliminary trials with our setup showed that 80% of control bees learned to associate hexane with food reward after 2–3 trials, the same level of learning exhibited by honey bees to other pure odorants (Matsumoto et al., 2012; Tan et al., 2015).

During acquisition training, the CS was paired with the unconditioned stimulus (US; 30% w/w pure unscented sucrose solution in a micropipette tip) as the reward. We lightly tapped one antenna with the US to elicit PER and then allowed the bee to feed. The US was presented 3 s after CS and overlapped with the CS for 2 s. A bee showing learning would extend its proboscis during the presentation of the CS only (response scored as all or none). We placed a fan 12 cm behind the bee and vented all odors out a window. We conditioned each bee six times with an inter-trial interval of 10 min, which facilitates honeybee olfactory learning (Menzel, 2001). During the memory tests, we exposed trained bees at each memory test time point to the CS alone (hexane) or to a novel odor (nonanal), none of which were rewarded (Menzel, 1999), such that half of the bees received the hexane followed by nonanal and half received nonanal followed by hexane. We calculated the Discrimination Index (DI) = response to the CS – response to novel odor. In total, we tested bee's memory at 1 h, 5 h, 24 h and 7 d after the last learning trial.

2.4.3. Treatments

We dissolved caffeine (CAS ID 58-08-2, Toronto Research Chemicals, Cat. No., C080100, ≥98.0% analytical purity, Canada) or artificial tea polyphenols (described above) prepared in 30% (w/w) analytical grade

sucrose and distilled water to make our test solution. The actual concentrations of the different TP components are shown in Table 1. In these learning experiments, we tested the efforts of two concentrations of caffeine (10 μg/ml and 100 μg/ml) and the same concentrations for TP. We chose these concentrations of caffeine because Wright et al. (2013) reported that honey bees can show improved learning and memory ability after collecting *Citrus* and *Coffea* nectar with a caffeine concentration <1 mM (194.19 μg/ml). The same concentrations were used for TP because they represent a wide range: a low TP concentration (10 μg/ml) and a higher TP concentration (100 μg/ml). As controls, we used separate groups of bees that were only fed pure 30% sucrose solution (w/w) containing now caffeine or TP. We first made the higher concentration solutions and then diluted them 10x with pure 30% sucrose solution (w/w) to obtain the lower concentrations.

We removed bees from the incubator on the morning of second day, harnessed them for our PER experiments, and allowed them to sit in the test environment for 5 h to acclimate. Bees were then individually fed once with a micropipette providing 10 μl of treatment. We then tested bees either 2 h after this acute exposure (testing short-term effects) or, with separate groups of bees, 1 d after exposure (testing longer term effects). For the 1 d bees, we exposed them to the treatment and then fed them to satiation with 30% pure sucrose at 9 pm of that day and kept them in an incubator (25 °C, 65% relative humidity) overnight.

With each bee, we also conducted an unrewarded memory test 7 d after the last learning trial. To do this, we removed bees from their PER stands after the 24 h memory test (see above) and placed them inside wood boxes (inside the incubator at 25 °C, 65% relative humidity). We fed each bee with 5 μl of 30% sucrose solution twice per day (at 9:00 a.m. and again at 9:00p.m.). On the sixth day, we fed bees in the morning, but did not feed them in the evening to ensure that they would be hungry for the 7 d memory test. This test consisted of with one presentation of the conditioned odor, hexane, and one presentation of the novel odor, nonanal, (both non-rewarded, presentation order alternated for half of the bees) on the following morning (7 d after the last learning trial).

2.5. Experiment 4. Effect of TP on honey bee antennal responses (EAG)

To test if TP could influence *A. mellifera* antennal response to alarm pheromone compounds, we recorded electroantennograms (EAG) of each bee to the same primary alarm compounds in honey bee sting alarm pheromone: isopentyl acetate (IPA), octyl acetate (OA), and benzyl acetate (BA) (Koeniger et al., 1979; Blum et al., 1978). We purchased our test compounds from Jingchun Biological Technology, Shanghai, China. After capturing honey bee foragers from entrances of three different colonies (sample sizes in Table S2), we then put them into cages and fed them different concentrations of TP (0 μg/ml, 10 μg/ml and 100 μg/ml) in 30% (w/w) sucrose solutions. We fed bees a single dose (in 10 μl) of TP and tested their EAG responses 2 h later.

In a preliminary test, we compared the responses of freshly dissected left and right antennae but found no difference between the responses and thereafter only used the left antennae. We cut off this antenna and placed it inside a glass electrode filled with insect Ringer's solution. The antenna was placed 1 cm away from the outlet of a polytetrafluoroethylene (PTFE) tube (1 cm inner diameter, 15 cm long) that provided the test odor in a constant air stream that was clean (500 ml active charcoal filtered) and humidified (distilled water, 90% relative humidity). All measurements were conducted at 25 °C. For each stimulation, we delivered an odor pulse for 3 s, mixing it into the continuous flow. To record antennal responses, we used a custom stimulus controller, a modified EAG amplifier (Wen et al., 2017a; 2017b) outputting a signal into a HP34405A Digital Multi Meter (Agilent, USA) and BenchVue software (Keysight, USA) running on a PC.

Each bee was exposed to only one level of TP (0, 10, or 100 μg/ml) and tested with one odor type (IPA, OA, or BA). Each bee was tested with the following ascending odor doses: 0 ng (blank control), 100 ng, 1000 ng, and 10,000 ng. The blank control was 5 μl of pure hexane (0 ng test odorant) and all subsequent doses were also provided in 5 μl of hexane. All test odors were pipetted onto clean filter paper (0.4 cm × 2.0 cm) placed inside a glass Pasteur pipette for delivery via the EAG system (see above). During testing, we provided the test odor for 3 s with an inter-trial interval of 30 s to provide sufficient recovery time (Wang et al., 2016).

2.6. Statistics

Our bioassay choice experiments consisted of three different arrays (0 vs. 10 μg/ml, 0 vs. 100 μg/ml, and 10 vs. 100 μg/ml). Each bee only experienced one kind of array, but made 10 trips to that array. Per bee, we therefore calculated the percentage of choices for the lower TP concentration feeder. We then generated a distribution of bee choices per array type and tested if the distribution means of these choices were significantly different from no preference (50%) using 2-sided Wilcoxon Signed-Rank tests.

We ran separate analyses for learning (PER) and memory (Discrimination Index). For memory, we examined each memory time point.

Our sample sizes ranged from 60 to 117 honey bee workers per treatment (Table S1) and we therefore used Repeated-Measures Mixed Models with a REML algorithm (bee identity is the repeated measure) to allow between group and within group comparisons (Matsumoto et al., 2012). We used sequential model simplification, first running all interactions, and then eliminating them if they were not significant. Tukey Honestly Significant Difference (HSD) tests were used to make corrected pairwise comparisons.

For the EAG experiment, we analyzed each alarm pheromone odor separately, using a Repeated-Measures Mixed Models with a REML algorithm and bee identity nested within odor type because each bee was tested with different concentrations of one type of odor. We log-transformed the EAG responses. We used sequential model simplification, first running all interactions, and then eliminating them if they were not significant. Tukey (HSD tests were used to make corrected pairwise comparisons. We used JMP Pro v13.0.0 (SAS Institute, USA) for all statistical analyses and show mean ± 95% CI (confidence interval) in our plots.

3. Results

3.1. Exp 1. Caffeine and TP within the tea nectar

Our collected tea nectar had a natural caffeine concentration of 15.83 ± 0.06 μg/ml (0.0792 mM, Fig. 1). Thus, the natural caffeine concentration of tea nectar is similar to the lower concentration of 10 μg caffeine/ml that we used.

Total tea polyphenols were a mixture of multiple compounds in the following average concentrations: 7.87 ± 0.38 μg/ml (0.0257 mM) gallocatechin (GA), 1.13 ± 0.07 μg/ml (0.0039 mM) epicatechin (EC), 9.18 ± 0.10 μg/ml (0.02 mM) epigallocatechin gallate (EGCG), and 0.921 ± 0.01 μg/ml (0.0021 mM) epicatechin gallate (ECG) (Fig. 1 and Table 1). We did not detect any epigallocatechin (EGC): 0 μg/ml (0 mM). This yields a total of 19.1 ± 0.56 μg/ml of TP compounds in natural tea nectar. We therefore prepared two different concentrations of TP compounds, all in the same proportions found in natural tea nectar, testing the effects of lower (10 μg TP/ml) and higher (100 μg TP/ml) concentration to bracket the natural concentrations.

3.2. Exp 2. Bioassay of forager choices for TP

Bees significantly preferred the TP feeder when given a choice between 0 and 10 μg/ml TP (62.9% of choices for TP, 2-tailed Wilcoxon Signed-Rank test, $W = 1163$, $P < 0.0001$) and between 0 and 100 μg/ml TP (63.3% of choices for the TP feeder, 2-tailed Wilcoxon Signed-Rank test, $W = 1249$, $P < 0.0001$. Fig. 2). However, when given a choice between 10 vs 100 μg/ml TP, foragers had no significant preference for

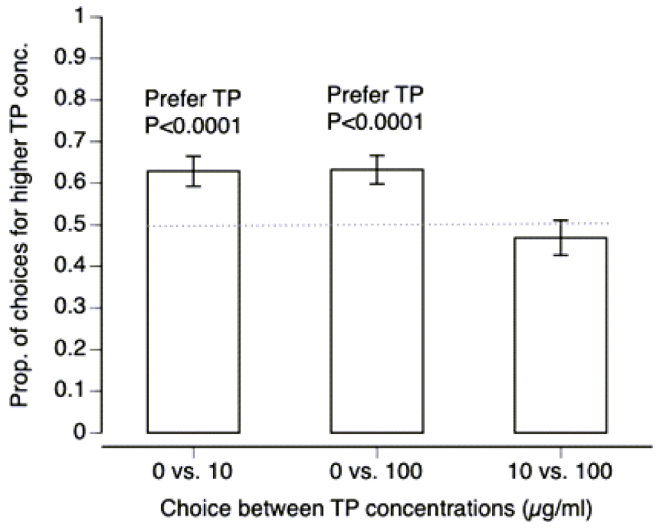

Fig. 2. Results of the TP paired-choice bioassay. The mean proportion of choices (out of 10 per bee) for the feeder with the higher TP concentration is shown (*P*-values from a 2-tailed Wilcoxon Signed-Rank test). Error bars indicate 95% confidence intervals. The dashed line shows the null hypothesis expectation of no preference. Bees preferred 10 and 100 μg/ml TP over the control but had no preference between 10 and 100 μg/ml TP.

either feeder (2-tailed Wilcoxon Signed-Rank test, $W = 321$, $P = 0.12$). Bees therefore preferred 10 and 100 μg/ml TP over the control.

3.3. Exp 3. Learning and memory

3.3.1. Effect of caffeine on learning

Bees learned (significant trial effect: $F_{5,4855} = 363.54$, $P < 0.0001$) and caffeine weakly improved learning (dose effect: $F_{2,969} = 4.44$, $P = 0.012$). The 100 μg/ml dose (each bee was fed 10 μl of this concentration) resulted in significantly higher learning than the control dose (Tukey HSD test, $P < 0.05$. Fig. 3A). There was no significant effect of treatment wait time (either 2 h or 1 d after treatment, $F_{1,969} = 0.07$, $P = 0.79$). However, there were significant effects of the interactions treatment wait time × trial ($F_{5,4855} = 25.16$, $P < 0.0001$) and trial × dose ($F_{10,4855} = 2.60$, $P = 0.004$). Caffeine did not increase learning in any individual trial (Tukey HSD test, $P > 0.05$). No other interactions were significant, and colony accounted for < 1% of model variance.

3.3.2. Effect of caffeine on memory

We note that nonanal may have potentially greater salience than hexane (Wright and Smith, 2004) for bees. However, an analysis of responses to the CS alone yielded similar results to the analysis of the DI. There was a significant effect of memory trial on memory retention, which declined over time ($F_{3,2657} = 7.97$, $P < 0.0001$, Fig. 3A). There were significant effects of treatment wait time ($F_{1,1175} = 12.23$, $P = 0.0005$) and dose ($F_{2,1366} = 37.80$, $P < 0.0001$). The interaction trial × dose ($F_{6,2685} = 2.35$, $P = 0.029$) was significant. The treatment wait time × dose was also significant ($F_{2,1170} = 8.45$, $P = 0.0002$), and caffeine improved memory retention (dose effect per bee): 2 h wait time (100 μg/ml better than the control dose) and 1 d (100 and 10 μg/ml better than control, Tukey HSD test, $P < 0.05$). Colony accounted for <1% of model variance (Fig. 3A).

3.3.3. Effect of TP on learning

As expected, bees learned in the TP trials (trial effect: $F_{5,4925} = 1016.86$, $P < 0.0001$). However, there was no significant effect of TP dose ($F_{2,981} = 1.78$, $P = 0.17$. Fig. 3B). Colony accounted for < 1% of model variance.

3.3.4. Effect of TP on memory

Memory significantly declined over 7 d ($F_{3,2690} = 13.37$, $P < 0.0001$, Fig. 3B), but TP increased memory retention (dose effect: $F_{2,11761} = 10.70$, $P < 0.0001$. Fig. 3B). A dose of 100 μgTP/ml (fed as 10 μl per bee) increased memory as compared to the control dose at the 5 h trial and the 24 h trial. In contrast, 10 μg TP/ml only increased memory to the control dose at the 5 h trial (Tukey HSD test, $P < 0.05$). The interaction treatment wait time × trial was significant ($F_{3,2692} = 3.47$, $P = 0.016$), but there were no significant differences between the effects of treatment wait time on memory at any tested time point (Tukey HSD test, $P > 0.05$). Colony accounted for <1% of model variance.

3.4. Exp 4. TP effect on EAG response to alarm pheromone components

For each alarm odor compound, bees fed 10 or 100 μg TP/ml generally had increased EAG responses as compared to bees fed the control treatment of 0 μg TP/ml (Fig. 4). Interestingly, this increase in EAG responsiveness occurred even in the absence of any test odor (hexane alone), suggesting that TP induces a general increase in antennal responsiveness. We therefore highlight exceptions to this trend below.

For IPA, there were significant effects of TP concentration ($F_{2,77} = 26.01$, $P < 0.0001$), odor concentration ($F_{3,153} = 491.31$, $P < 0.0001$), and the interaction TP concentration × odor concentration ($F_{6,153} = 4.59$, $P = 0.0003$). Colony accounted for < 1% of model variance. When presented with 10000 ng of IPA, control bees and bees fed 100 μg/ml TPA did not have significantly different EAG responses.

For OA, there were also significant effects of TP concentration ($F_{2,91} = 35.70$, $P < 0.0001$), odor concentration ($F_{3,153} = 711.43$, $P < 0.0001$), and the interaction TP concentration × odor concentration ($F_{6,153} = 7.46$, $P < 0.0001$). Colony accounted for < 1% of model variance. When presented with 1000 ng of OA, control bees and bees fed 10 μg/ml TP did not have significantly different EAG responses.

For BA, TP concentration ($F_{2,82} = 39.07$, $P < 0.0001$), odor concentration ($F_{3,153} = 1019.33$, $P < 0.0001$), and the interaction TP concentration × odor concentration ($F_{6,153} = 4.40$, $P = 0.0004$) were also all significant. Colony accounted for < 1% of model variance.

4. Discussion

We provide the first evidence that bees prefer nectar with tea polyphenols (TP) over control nectar at natural and elevated TP concentrations. In addition, TP can affect bee olfactory cognitive ability and olfactory sensitivity. Caffeine, but not TP, improved learning. Since bees can be exposed to these compounds and immediately begin to learn or experience a longer delay before learning, we tested the effects of exposure 2 h or 1 d before learning. The effects of exposure time delay before learning were complex and varied depending upon the learning trial and compound (caffeine or TP). However, both caffeine and TP significantly improved memory retention and, in general, more recent treatment (2 h) resulted in better retention than treatment 1 d before. TP also elevated antennal responsiveness to tested odors.

Our chemical analyses of natural tea nectar revealed an average caffeine concentration of 0.079 mM, within the range reported by Wright et al. (2013) for *Coffea* and *Citrus* (0.003 mM-0.253 mM). In tea nectar, we found that caffeine (0.0792 mM) was >8-fold more concentrated than TP (0.0096 mM), a result that agrees with prior studies showing that tea polyphenols are concentrated in the young leaves of *C. sinensis*, but occur in lower concentrations in its nectar (Sharma et al., 1986). These data also support prior research demonstrating that caffeine and TP concentrations differ depending upon the part of the tea plant analyzed (Morikawa et al., 2013; Lin et al., 2003). We identified similar TP compounds in nectar and tea leaves, except for EGC, which is one of the most abundant TP components in young tea leaves (Graham, 1992). We found an average of 19.1 μg/ml of total TP compounds in tea nectar, a concentration between our two test concentrations of 10 μg TP/

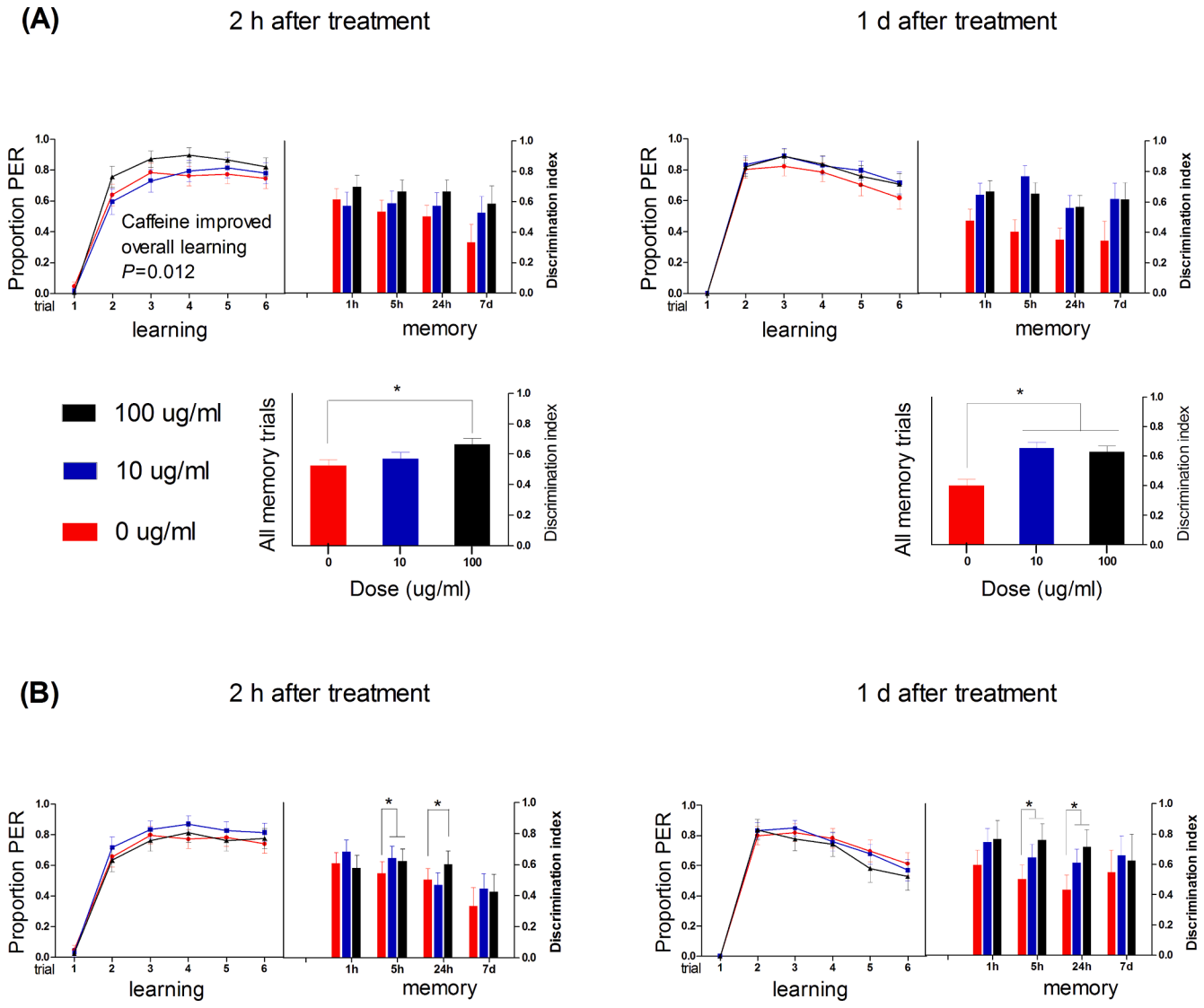

Fig. 3. Effect of caffeine and TP on bee learning and memory when tested 2 h or 1 d after feeding on the treatment. (A) Bees trained 2 h ($n_{0\ \mu g/ml} = 78$, $n_{10\ \mu g/ml} = 87$, $n_{100\ \mu g/ml} = 75$) after feeding on caffeine had improved learning ($P = 0.012$), but not if they were trained 1 d ($n_{0\ \mu g/ml} = 72$, $n_{10\ \mu g/ml} = 87$, $n_{100\mu g/ml} = 78$) after feeding on caffeine. The plots below pool the data from all memory trials and show that there were significant effects of caffeine at both treatment wait times (Tukey HSD test, $^*P < 0.05$). (B) TP did not improve learning 2 h after feeding ($n_{0\ \mu g/ml} = 78$, $n_{10\ \mu g/ml} = 117$, $n_{100\ \mu g/ml} = 87$) or 1 d after feeding ($n_{0\ \mu g/ml} = 75$, $n_{10\ \mu g/ml} = 93$, $n_{100\ \mu g/ml} = 60$). TP improved memory (Tukey HSD test, $^*P < 0.05$). All plots show mean ± 95% confidence intervals.

ml and 100 μg TP/ml.

Interestingly, foragers preferred nectar with TP at concentrations at (10 μg/ml) and above (100 μg/ml) what is found in nature (Fig. 2). Singaravelan et al. (2005) showed that a low caffeine concentration in nectar (25 ppm in their natural caffeine range test) can create a pollinator feeding preference. The reasons for these preferences remain unclear, but Kucharski and Maleszka (2005) reported that caffeine can alter honey bee gene expression patterns in the brain. Caffeine is an adenosine receptor antagonist and improved responses of mushroom body neurons involved in olfactory learning and memory (Wright et al., 2013). In our study, honey bee memory improved overall when bees fed more recently on caffeine and TP (within 2 h as compared to 1 d before the first learning trial). TP and caffeine improved memory retention, and caffeine weakly improved learning. The tea plant may therefore benefit from these pollinator effects.

TP consumption generally increased EAG responsiveness, even in the absence of test odors. This was not true in all cases (Fig. 4), but the trend is sufficiently strong to suggest that additional studies are required. Does this heightened EAG responsiveness translate into an ability to discriminate odors or is there simply a heighted basal activity level that does not enhance overall responses to odors? If the former is correct, there are implications for plant fitness. Honey bees will avoid floral resources marked with alarm pheromone (Wen et al., 2017a; 2017b). If bees that have fed upon TP in nectar have heightened sensitivity to alarm odors, this could result in an increased spatial area in which bees avoid alarm pheromones, reducing honey bee visitation of tea inflorescences upon which foragers had previously released sting alarm pheromone. Such reduced floral visitation is known to decrease plant fitness by decreasing pollination and seed set (Romero et al., 2011).

However, *C. sinensis* could also gain from the forager attraction for nectar with TP. The push and pull of these different forces on the coevolution between *C. sinensis* and its pollinators would be useful to explore in general, particularly since these compounds likely occur in nectar as a side effect of their anti-herbivore effects in general plant tissue. TP compounds may also occur as defensive compounds in other plant species, a point for further investigation. Many different pollinators and their plants face similar issues with the anti-herbivory compounds that plants have evolved. The evolutionary and theoretical

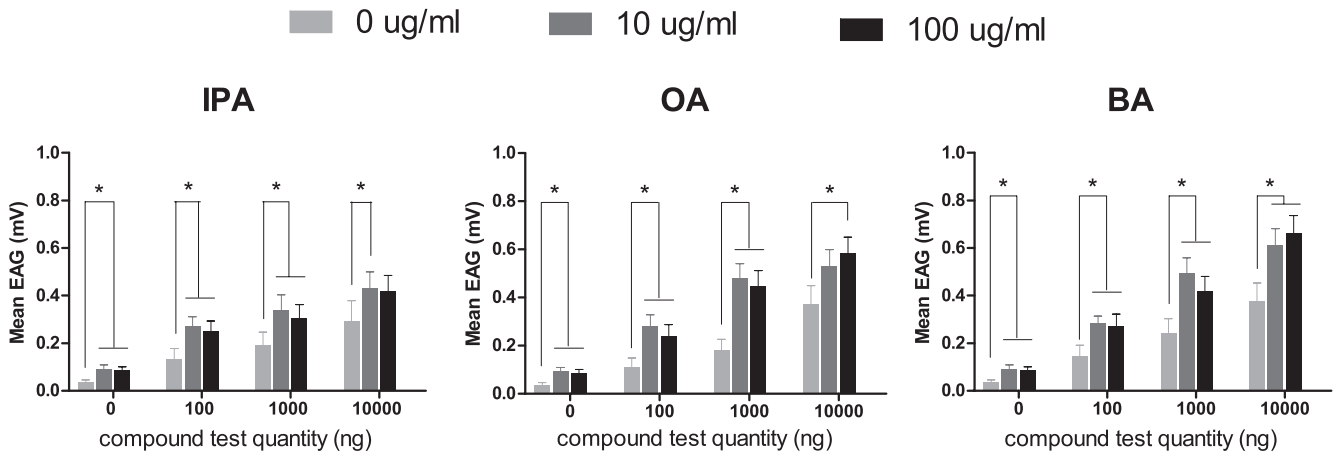

Fig. 4. Honey bee electroantennogram (EAG) responses (absolute values shown) to major alarm pheromone components after consuming TP (0, 10 or 100 μg/ml) 2 h before testing. We tested EAG responses to isopentyl acetate (IPA. $n_{0\mu g/ml\text{-}0ng} = 22$, $n_{0\mu g/ml\text{-}100ng} = 22$, $n_{0\mu g/ml\text{-}1000ng} = 22$, $n_{0\mu g/ml\text{-}10000ng} = 22$; $n_{10\mu g/ml\text{-}0ng} = 24$, $n_{10\mu g/ml\text{-}100ng} = 24$, $n_{10\mu g/ml\text{-}1000ng} = 24$, $n_{10\mu g/ml\text{-}10000ng} = 24$; $n_{100\mu g/ml\text{-}0ng} = 23$, $n_{100\mu g/ml\text{-}100ng} = 23$, $n_{100\mu g/ml\text{-}1000ng} = 23$, $n_{100\mu g/ml\text{-}10000ng} = 23$), octyl acetate (OA. $n_{0\mu g/ml\text{-}0ng} = 23$, $n_{0\mu g/ml\text{-}100ng} = 23$, $n_{0\mu g/ml\text{-}1000ng} = 23$, $n_{0\mu g/ml\text{-}10000ng} = 23$; $n_{10\mu g/ml\text{-}0ng} = 24$, $n_{10\mu g/ml\text{-}0ng} = 23$, $n_{10\mu g/ml\text{-}1000ng} = 23$, $n_{10\mu g/ml\text{-}10000ng} = 23$; $n_{100\mu g/ml\text{-}0ng} = 23$, $n_{100\mu g/ml\text{-}100ng} = 23$, $n_{100\mu g/ml\text{-}1000ng} = 23$, $n_{100\mu g/ml\text{-}10000ng} = 23$), and benzyl acetate (BA. $n_{0\mu g/ml\text{-}0ng} = 22$, $n_{0\mu g/ml\text{-}100ng} = 22$, $n_{0\mu g/ml\text{-}1000ng} = 22$, $n_{0\mu g/ml\text{-}10000ng} = 22$; $n_{10\mu g/ml\text{-}0ng} = 23$, $n_{10\mu g/ml\text{-}100ng} = 23$, $n_{10\mu g/ml\text{-}1000ng} = 23$, $n_{10\mu g/ml\text{-}10000ng} = 23$; $n_{100\mu g/ml\text{-}0ng} = 23$, $n_{100\mu g/ml\text{-}100ng} = 24$, $n_{100\mu g/ml\text{-}1000ng} = 24$, $n_{100\mu g/ml\text{-}10000ng} = 24$). TP improved antennal responsiveness (Tukey HSD test, $^*P < 0.05$). All plots show mean ± 95% confidence intervals.

consequences of such spandrels, phenotypic traits such as defensive nectar compounds that are byproducts with respect to pollinators rather than the result of adaptive selection to harm or influence pollinators, should be better understood.

5. Data availability

All data are available at Zenodo.org at https://doi.org/10.5281/zenodo.4312035.

CRediT authorship contribution statement

Zhiwen Gong: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Gaoying Gu:** Investigation, Writing - review & editing. **Yuan Wang:** Methodology, Investigation. **Shihao Dong:** Methodology, Validation, Investigation, Writing - review & editing. **Ken Tan:** Conceptualization, Methodology, Validation, Investigation, Resources, Supervision, Project administration, Funding acquisition. **James C. Nieh:** Conceptualization, Methodology, Validation, Writing - review & editing, Formal analysis, Visualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary tables for this article can be found online at https://doi.org/10.1016/j.jinsphys.2020.104177.

References

Bitterman, M., Menzel, R., Andrea, F., Sabine, S., 1983. Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). J. Comp. Psychol. 97, 107–119.
Blum, M.S., Fales, H.M., Tucker, K.W., Collins, A.M., 1978. Chemistry of the sting apparatus of the worker honeybee. J. Apic. Res. 17, 218–221.
Couvillon, M.J., Toufailia, A.H., Butterfield, T.M., Schrell, F., Ratnieks, F.L.W., Schürch, R., 2015. Caffeinated forage tricks honeybees into increasing foraging and recruitment behaviors. Curr. Biol. 25, 2815–2818.
Gegear, R.J., Manson, J.S., Thomson, J.D., 2007. Ecological context influences pollinator deterrence by alkaloids in floral nectar. Ecol. Lett. 10, 375–382.
Giurfa, M., Sandoz, J.C., 2012. Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees. Learn. Mem. 19, 54–66.
Gong, Z., Wang, C., Nieh, J.C., Tan, K., 2016. Inhibiting DNA methylation alters olfactory extinction but not acquisition learning in *Apis cerana* and *Apis mellifera*. J. Ins. Physiol. 90, 43–48.
Gong, Z., Tan, K., Nieh, J.C., 2018. First demonstration of olfactory learning and long term memory in honey bee queens. J. Exp. Biol. 221, jeb177303.
Graham, H.N., 1992. Green tea composition, consumption, and polyphenol chemistry. Prev. Med. 21, 334–350.
Harborne, J.B., 1993. Introduction To Ecological Biochemistry, 4th ed. Academic Press.
Irwin, R.E., Cook, D., Richardson, L.L., Manson, J.S., Gardner, D.R.l., 2014. Secondary compounds in floral rewards of toxic rangeland plants: impacts on pollinators. J. Agric. Food Chem., 62, 7335-7344.
Jacobsen, D.J., Raguso, R.A., 2018. Lingering effects of herbivory and plant defenses on pollinators. Curr. Biol. 28, R1164–R1169.
Jones, P.L., Agrawal, A.A., 2016. Consequences of toxic secondary compounds in nectar for mutualist bees and antagonist butterflies. Ecology 97, 2570–2579.
Koeniger, N., Weiss, J., Maschwitz, U., 1979. Alarm pheromones of the sting in the genus Apis. J. Insec. Physiol. 25, 467–476.
Kucharski, R., Maleszka, R., 2005. Microarray and real-time PCR analyses of gene expression in the honeybee brain following caffeine treatment. J. Molec. Neurosci. 27, 269–276.
Lin, Y.S., Wu, S.S., Lin, J.K., 2003. Determination of tea polyphenols and caffeine in tea flowers (*Camellia sinensis*) and their hydroxyl radical scavenging and nitric oxide suppressing effects. J. Agric. Food Chem. 51, 975–980.
Matsumoto, Y., Sandoz, J.C., Giurfa, M., 2012. Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: a step toward standardized procedures. J. Neurosci. Meth. 211, 159–167.
Menzel, R., 1999. Memory dynamics in the honeybee. J. Comp. Physiol. A 185, 323–340.
Menzel, R., 2001. Searching for the memory trace in a mini-brain, the honeybee. Learn. Mem. 8, 53–62.
Morikawa, T., Ninomiya, K., Miyake, S., Miki, Y., Okamoto, M., Yoshikawa, M., Muraoka, O., 2013. Flavonol glycosides with lipid accumulation inhibitory activity and simultaneous quantitative analysis of 15 polyphenols and caffeine in the flower buds of *Camelliasinensis* from different regions by LCMS. Food Chem. 140, 353–360.

Mustard, J.A., Dews, L., Brugato, A., Dey, K., Wright, G.A., 2012. Consumption of an acute dose of caffeine reduces acquisition but not memory in the honey bee. Behav. Brain Res. 232, 217–224.
Romero, G.Q., Antiqueira, P.A.P., Koricheva, J., 2011. A meta-analysis of predation risk effects on pollinator behaviour. PLoS One 6, e20689.
Sharma, O.P., Raj, D., Garg, R., 1986. Toxicity of nectar of tea (*Camellia thea* L.) to honeybees. J. Apic. Res. 25, 106–108.
Si, A., Zhang, S.W., Maleszka, R., 2005. Effects of caffeine on olfactory and visual learning in the honey bee (*Apis mellifera*). Pharm. Biochem. Behav. 82, 664–672.
Singaravelan, N., Nee'man, G., Inbar, M., Izhaki I., 2005. Feeding responses of free-flying honeybees to secondary compounds mimicking floral nectars. J. Chem. Eco. 31: 2791–2804.
Stevenson, P.C., Nicolson, S.W., Wright, G.A., 2017. Plant secondary metabolites in nectar: impacts on pollinators and ecological functions. Func. Ecol. 31, 65–75.
Sullivan, R.J., Hagen, E.H., Hammerstein, P., 2008. Revealing the paradox of drug reward in human evolution. Pro. Roy. Soc. B. 275, 1231.
Tan, K., Chen, W., Dong, S., Liu, X., Wang, Y., Nieh, J.C., 2015. A neonicotinoid impairs olfactory learning in Asian honey bees (*Apis cerana*) exposed as larvae or as adults. Scientific. Rep. 5, 10989.
Vincent, D., Ellis, J.D., Neumann, P., 2013. The Colossbeebook volume I: Standard methods for Apis mellifera research. International Bee Research Association IBRA. V52.
Wang, Z., Wen, P., Qu, Y., Dong, S., Li, J., Tan, K., Nieh, J.C., 2016. Bees eavesdrop upon informative and persistent signal compounds in alarm pheromones. Scientific Rep. 6, 25693.
Wen, P., Cheng, Y., Wang, Z., Tan, K., Nieh, J.C., 2017a. The sex pheromone of a globally invasive honey bee predator, the Asian eusocial hornet, *Vespa velutina*. Scientific Rep. 7, 12956.
Wen, P., Cheng, Y.N., Qu, Y.F., Zhang, H.X., Li, J.J., Heather, B., Tan, K., Nieh, J., 2017b. Foragers of sympatric Asian honey bee species intercept competitor signals by avoiding benzyl acetate from *Apis cerana* alarm pheromone. Scientific Rep. 7, 6721.
Wright, G.A., Smith, B.H., 2004. Different thresholds for detection and discrimination of odors in the honey bee (*Apis mellifera*). Chem. Senses 29, 127–135.
Wright, G.A., Baker, D.D., Palmer, M.J., Stabler, D., Mustard, J.A., Power, E.F., Borland, A.M., Stevenson, P.C., 2013. Caffeine in floral nectar enhances a pollinator's memory of reward. Science 339, 1202–1204.
Yang, G.H., 2005. Harm of introducing the western honey bee *Apis mellifera* L. to the Chinese *Apis cerana* F. and its ecological impact. Acta Entomol. Sinica 48, 401–406.
Zhang, J., Wang, Z., Wen, P., Qu, Y., Tan, K., Nieh, J.C., 2018. The reluctant visitor: a terpenoid in toxic nectar can reduce olfactory learning and memory in Asian honey bees. J. Exp. Biol. 221, jeb168344.
Zhou, J.H., Yao, L.H., Li, H., Chen, L.Z., Wu, L.M., Zhao, J., 2013. Floral classification of honey using liquid chromatography-diode array detection-trade mass spectrometry and chemometric analysis. Food Chem. 145, 941–949.