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Caffeine in floral nectar enhances a pollinator's memory of reward

G. A. Wright[1], D. D. Baker[2], M. J. Palmer[3], D. Stabler[1&2], J. A. Mustard[4], E. F. Power[1&2], A. M. Borland[2], and P. C. Stevenson[5&6]

[1]Centre for Behaviour and Evolution, Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK; [2]School of Biology, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK; [3]Division of Neuroscience, Medical Research Institute, Ninewells Medical School, University of Dundee, Dundee, DD1 9SY, UK; [4]School of Life Sciences, Arizona State University, Tempe, AZ; [5]Jodrell Laboratory, Royal Botanic Gardens, Kew, Surrey, TW9 3AB, UK; [6]Natural Resources Institute, University of Greenwich, Chatham, Kent, ME4 4TB, UK

Abstract

Plant defence compounds occur in floral nectar, but their ecological role is not well-understood. We provide the first evidence that plant compounds pharmacologically alter pollinator behaviour by enhancing their memory of reward. Honeybees rewarded with caffeine, which occurs naturally in nectar of *Coffea* and *Citrus* species, were three times more likely to remember a learned floral scent than those rewarded with sucrose alone. Caffeine potentiated responses of mushroom body neurons involved in olfactory learning and memory by acting as an adenosine receptor antagonist. Caffeine concentrations in nectar never exceeded the bees' bitter taste threshold, implying that pollinators impose selection for nectar that is pharmacologically active but not repellent. By using a drug to enhance memories of reward, plants secure pollinator fidelity and improve reproductive success.

Many drugs commonly consumed by humans are produced by plants as a form of toxic defence against herbivores (*1, 2*). While plant-derived drugs like caffeine or nicotine are lethal in high doses (*3-5*), they have pharmacological effects at low doses that affect mammalian behaviour. For example, low doses of caffeine are mildly rewarding and enhance cognitive performance and memory retention (*6*).

Interestingly, caffeine has been detected in low doses in the floral nectar and pollen of *Citrus* (*7*), but whether it has an ecological function is unknown.

Two caffeine-producing plant genera, *Citrus* and *Coffea*, have large floral displays with strong scents and produce more fruits and seeds when pollinated by bees (*8, 9*). If caffeine confers a selective advantage when these plants interact with pollinators, we might expect it to be commonly encountered in nectar. We measured caffeine in the nectar of 3 species of *Coffea* (*C. canephora*, *C. arabica*, and *C. liberica*) and 4 species of *Citrus* (*C. paradisi*, *C. maxima*, *C. sinesis*, *C. reticulata*) using liquid chromatography-mass spectrometry (*10*, Fig. S1A). When caffeine was present, its concentration ranged from 0.003 - 0.253 mM. The median caffeine concentration in both genera was not significantly different (Fig. 1A, Mann-Whitney, $Z = -1.09$, $P = 0.272$). Caffeine was more common in the nectar of *C. canephora* than in *C. arabica* or *C. liberica* (*Coffea*: logistic regression $\chi_2^2 = 11.1$, $P = 0.004$); it was always present in *Citrus* nectar. The mean total nectar sugar concentration ranged from 0.338-0.843 M (Fig. 1B, see Fig S1B for individual sugars). Caffeine concentration in nectar did not correlate with total sugar concentration (Pearson's $r = 0.063$, $P = 0.596$).

We hypothesized that caffeine could affect the learning and memory of foraging pollinators. To test this, we trained individual honeybees to associate floral scent with 0.7 M sucrose and 7 different concentrations of caffeine and tested their olfactory memory. Using a method for classical conditioning of feeding responses (proboscis extension reflex, *11*), bees were trained for 6 trials with 30 s between each pairing of odour with reward. This inter-trial interval approximated the rate of floral visitation exhibited by honeybees foraging from multiple flowers on a single *Citrus* tree (see methods). The presence of low doses of caffeine in reward had a weak effect on the rate of learning (Fig. 2A), but it had a profound effect on long-term memory. When rewarded with solutions containing nectar-levels of caffeine, three times as many bees remembered the conditioned scent 24 h later and to responded as if it predicted reward (Fig. 2B, logistic regression, $\chi_7^2 = 41.9$, $P < 0.001$). Twice as many bees remembered it 72 h later (Fig. 2C). This improvement in memory performance was not due to a general increase in olfactory

sensitivity resulting from caffeine consumption (Fig. S2A). In fact, the effect of caffeine on long-term olfactory memory in bees was greater than that produced by high concentrations of sucrose when the same experimental methods were used (e.g. 2.0 M, Fig. S2B).

Caffeine's influence on cognition in mammals is in part mediated by its action as an adenosine receptor antagonist (*11*). In the hippocampal CA2 region, inhibition of adenosine receptors by caffeine induces long-term potentiation (*12*), a key mechanism of memory formation (*13*). The Kenyon cells (KCs) in mushroom bodies of the insect brain are similar in function to hippocampal neurons: they integrate sensory input during associative learning, exhibit long-term potentiation and are involved in memory formation (*14-16*). To test whether nectar-caffeine doses affect mushroom body function, we made whole-KC recordings in the intact honeybee brain. Caffeine (100 μM) evoked a small increase in the holding current (I_M) and depolarized KC membrane potential (V_M) towards the action potential firing threshold, by increasing nicotinic ACh receptor (nAChR) activation (Fig. 3A-D). To test whether the observed effects of caffeine were due to interactions with adenosine receptors, we applied the adenosine receptor antagonist, DPCPX, and observed that it similarly increased I_M and depolarized V_M but to a lesser extent (Fig. 3E,F). Both caffeine and DPCPX affected KC response kinetics evoked by brief, local application of ACh, increasing the activation rate and slowing the decay (Fig.3G,H). Our data show that caffeine modulates cholinergic input via a postsynaptic action, but could act via presynaptic adenosine receptors to potentiate ACh release (*17*). The resulting increase in KC excitability should lead to an increased probability of action potential firing in response to sensory stimulation (*18*), thereby facilitating the induction of associative synaptic plasticity in KCs (*19*). The enhanced activation of KCs may also facilitate plasticity at synapses with mushroom body extrinsic neurons (*20*), which exhibit spike-timing-dependent plasticity (*21*). In this way, a 'memory trace' could be formed for the odour associated with reward during and after conditioning (*22-23*).

Caffeine is bitter tasting to mammals and is both toxic (*24*) and repellent to honeybees at high concentrations (*25, 26*). If bees can detect caffeine, they might learn to avoid flowers offering nectar

containing it (*27*). We found that honeybees were deterred from drinking sucrose solutions containing caffeine at concentrations greater than 1 mM (Fig. 4); they also have neurons that detect caffeine in sensilla on their mouthparts (Fig. S3). However, nectar concentrations never exceeded 0.3mM (i.e. 0.058 mg/ml), even though levels of caffeine in vegetative and seed tissues of *Coffea* have been reported to be as great as 24mg/ml (*28*). This implies that pollinators drive selection towards concentrations of caffeine that are not repellent but still pharmacologically active.

Our data show that plant-produced alkaloids like caffeine have a role in addition to defence: they can pharmacologically manipulate a pollinator's behaviour. When bees and other pollinators learn to associate floral scent with food while foraging (*29*), they are more likely to visit flowers bearing the same scent signals. Such behaviour increases their foraging efficiency (*30*) while concomitantly leading to more effective pollination (*31, 32*). Our experiments suggest that by affecting a pollinator's memory, plants reap the reproductive benefits arising from enhanced pollinator fidelity.

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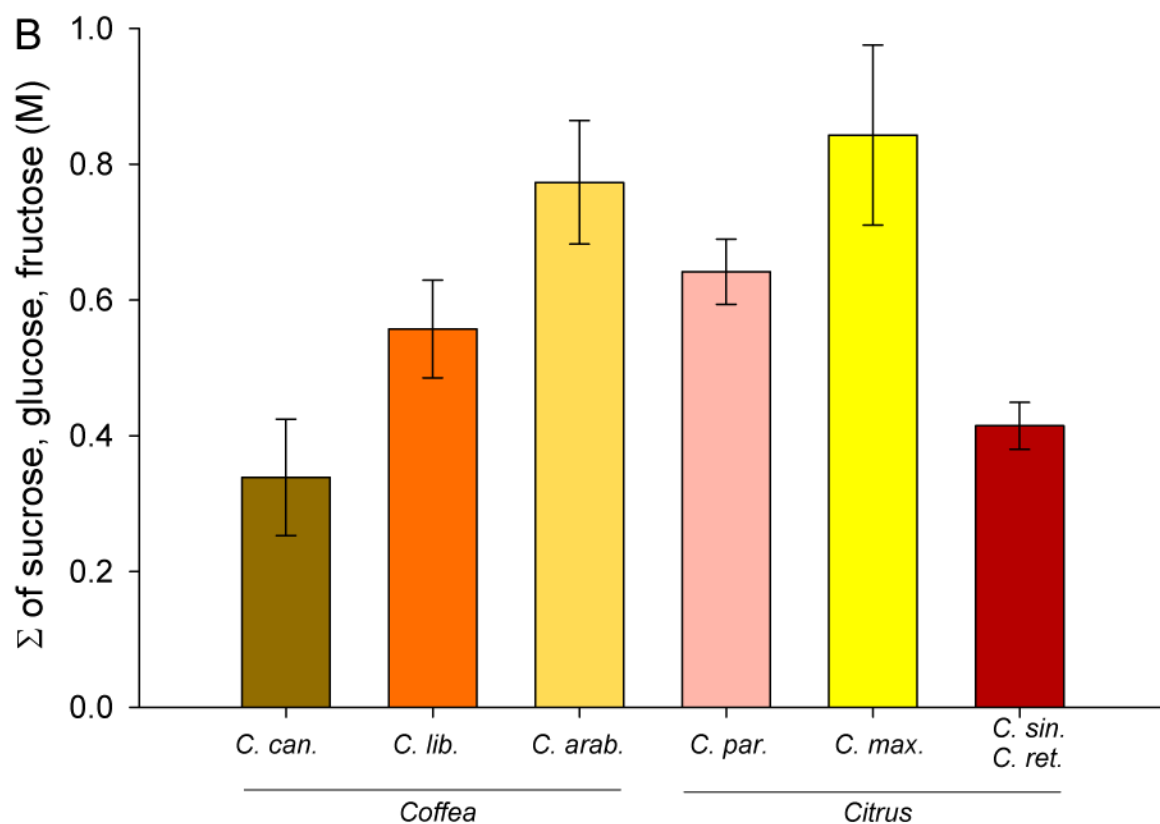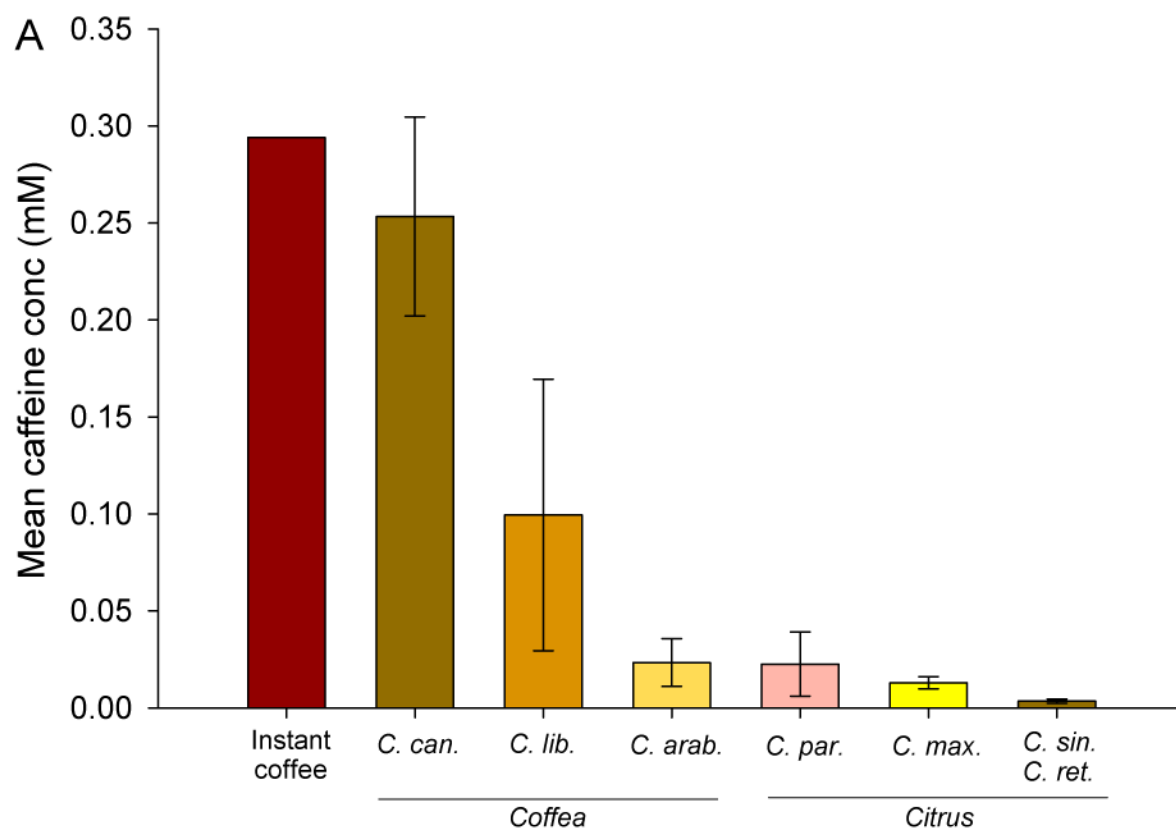
Figure 1. (**A**) Caffeine concentration in *Coffea* and *Citrus sp.* and a cup of instant coffee. Caffeine concentration depended on species within each genus (*Coffea*: Kruskal-Wallis, χ_2^2 = 28.1, P < 0.001; *Citrus*: Kruskal-Wallis, χ_2^2 = 6.98, P = 0.030); *C. canephora* had the highest mean concentration of all species sampled. (**B**) The sum of the concentration of sucrose, glucose and fructose (total nectar sugars) depended on species (1-way ANOVA: $F_{5, 161}$ = 4.64, P < 0.001) and was greatest in *Citrus maxima* and hybrids (citron, lemons, clementines). (*C. can.* = *Coffea canephora,* N = 34; *C. lib.* = *Coffea liberica*, N = 31; *C. arab.* = *Coffea arabica*, N = 27; *C. par.* = *Citrus paradisi* and hybrids, N_{cp} = 17; *C. max.* = *Citrus maxima* and hybrids, N = 5; *C. sin.* and *C. ret.* = *Citrus sinensis* and *Citrus reticulata,* N_{CS} = 7, N_{CR} = 5 – data for these two species was pooled).
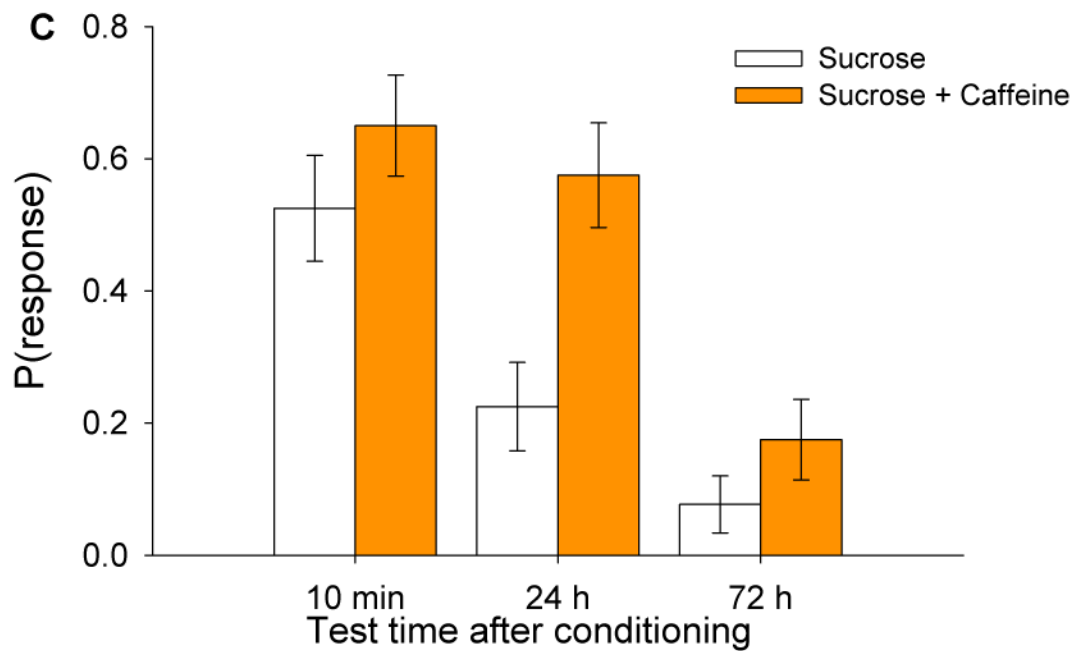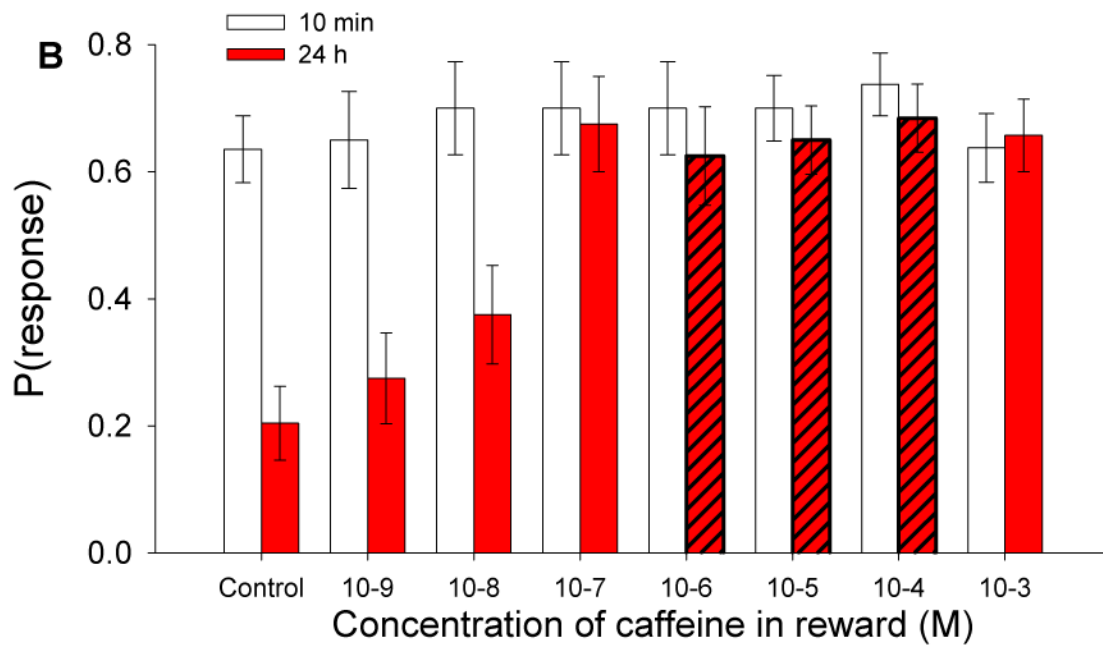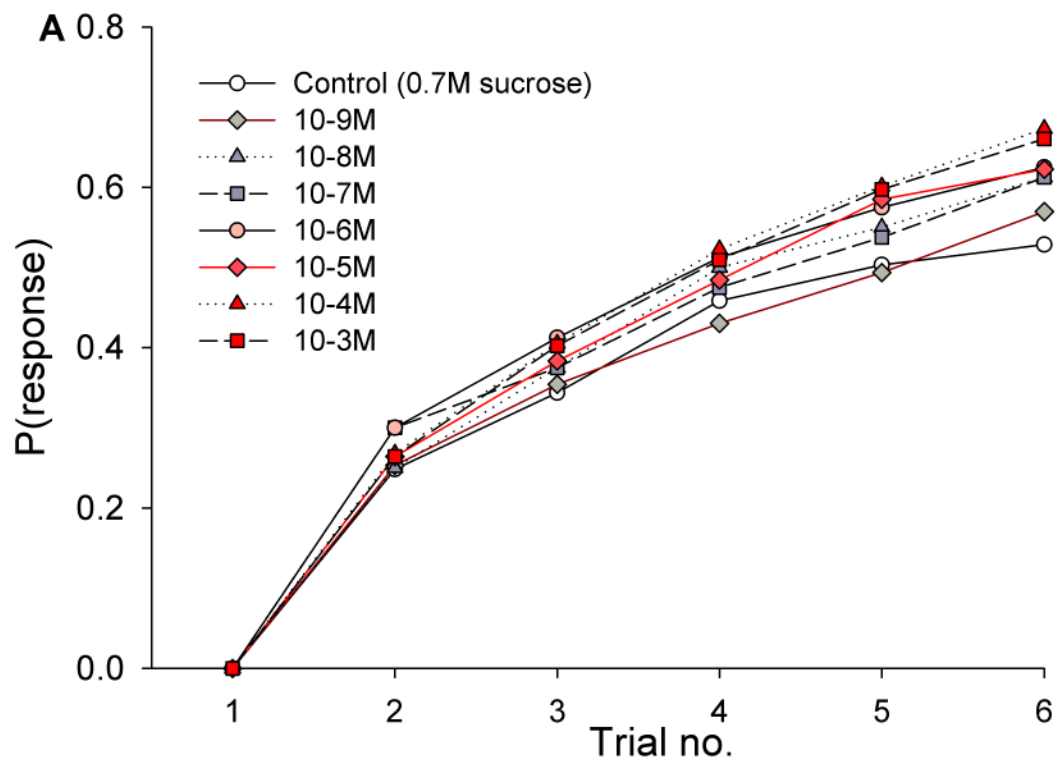
Figure 2. (**A**) The rate of learning of bees conditioned with an odour stimulus paired with a 0.7 M sucrose reward containing caffeine. The rate of learning was slightly greater for the bees fed caffeine in reward during conditioning (logistic regression, χ_1^2 = 4.85, P = 0.028). N $\geq$ 79 for all groups. (**B**) Memory recall test for odours at 10 min (clear bars) or 24 h (red bars) after bees had been trained as in (A). Bright red bars indicate that the response at 24 h was significantly different from the control (0.7 M sucrose) (least-squares contrasts: P < 0.05); dark red bars were not significantly different. Nectar-levels of caffeine are indicated by hatching. N > 79 for each group. (**C**) Bees fed 0.1 mM caffeine in sucrose (red bars) were more likely to remember the conditioned odour sucrose alone (white bars) (logistic regression, χ_1^2 = 9.04, P < 0.003) at 24 h and 72 h after conditioning. N = 40 per group.

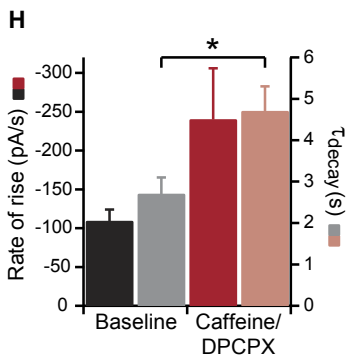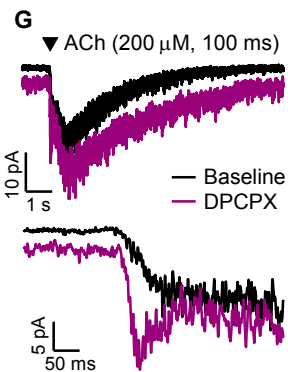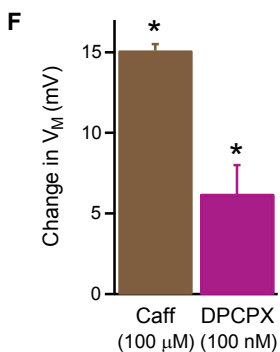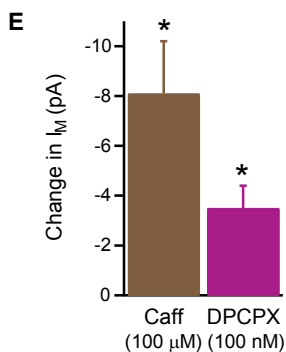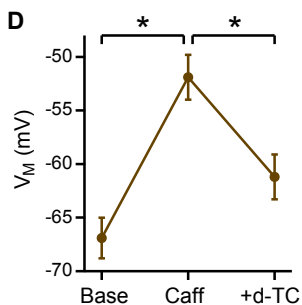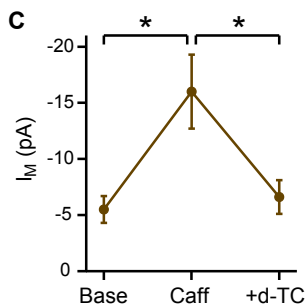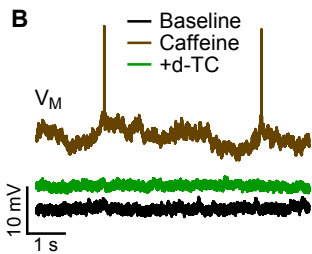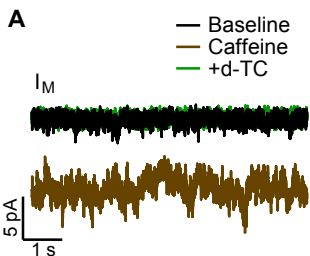**Figure 3**. The effect of caffeine on Kenyon cells. (**A, B**) Example traces from a KC in intact honeybee brain recorded under voltage-clamp (**A**, V_H = -73 mV) and current-clamp (**B**; at resting V_M), showing the increase in I_M and depolarization evoked by bath application of caffeine (100 μM), and subsequent reversal by the nAChR antagonist d-TC (500 μM). (**C, D**) Mean data showing the reversal by d-TC (500 μM) of the effect of caffeine (Caff; 100 μM) on I_M (**C**; N = 6, t_5 = 4.03, P = 0.010; t_5 = 4.07, P = 0.010) and V_M (**D**; N = 6, t_5 = 34.1, P < 0.001; t_5 = 12.0, P < 0.001). (**E, F**) Comparison of the mean effects of caffeine and DPCPX on I_M (**E**, Caff: N = 10, t_9 = 3.84, P = 0.004; DPCPX: N = 6, t_5 = 4.04, P = 0.010) and V_M (**F**, Caff: N = 6, t_5 = 34.1, P < 0.001; DPCPX: N = 6, t_5 = 3.39, P = 0.019). (**G, H**) Example traces (**G**; rising phase shown on an expanded time-scale below) and mean data (**H**, Rate of rise: N = 6, t_5 = 2.20, P = 0.079; τ_{decay}: N = 9, t_8 = 3.54, P = 0.008) showing that DPCPX (100 nM) and caffeine (100 μM) slowed the decay and, in 6 of 9 KCs, potentiated the fast component of the response evoked by exogenous ACh. (Student's paired t-test used in all comparisons).

Figure 4. Bees are more likely to reject sucrose solutions containing caffeine of concentrations greater than 1 mM (logistic regression, χ_4^2 = 23.4, P < 0.001; for 0.7M and 1.0M, 1 mM caffeine vs sucrose *post hoc*, P < 0.05; for 0.3M, 100 mM caffeine vs sucrose *post hoc*, P < 0.05). Bees were less likely to drink 0.3 M sucrose (pale pink diamonds) than 0.7M (pink circles) or 1.0M solutions (red triangles) (logistic regression, χ_2^2 = 8.69, P = 0.013). Mean responses ± SE. $N_{0.3M}$ = 29, $N_{0.7M}$ = 100, $N_{1.0M}$ = 20.

A

Mean caffeine conc (mM)

Instant coffee · *C. can.* · *C. lib.* · *C. arab.* · *C. par.* · *C. max.* · *C. sin.* *C. ret.*

Coffea · *Citrus*

B

Σ of sucrose, glucose, fructose (M)

C. can. · *C. lib.* · *C. arab.* · *C. par.* · *C. max.* · *C. sin.* *C. ret.*

Coffea · *Citrus*

A

P(response) vs Concentration of caffeine (M) in 0.7 M sucrose reward solution

Legend: CS, Novel

x-axis labels: Control, 10-9, 10-8, 10-7, 10-6, 10-5, 10-4, 10-3

B

P(response) vs Reward concentration

Legend: 10min, 24h

x-axis labels: 0.3M, 1.0M, 2.0M

A. Sucrose

B. Caffeine

C. Sucrose + Caffeine

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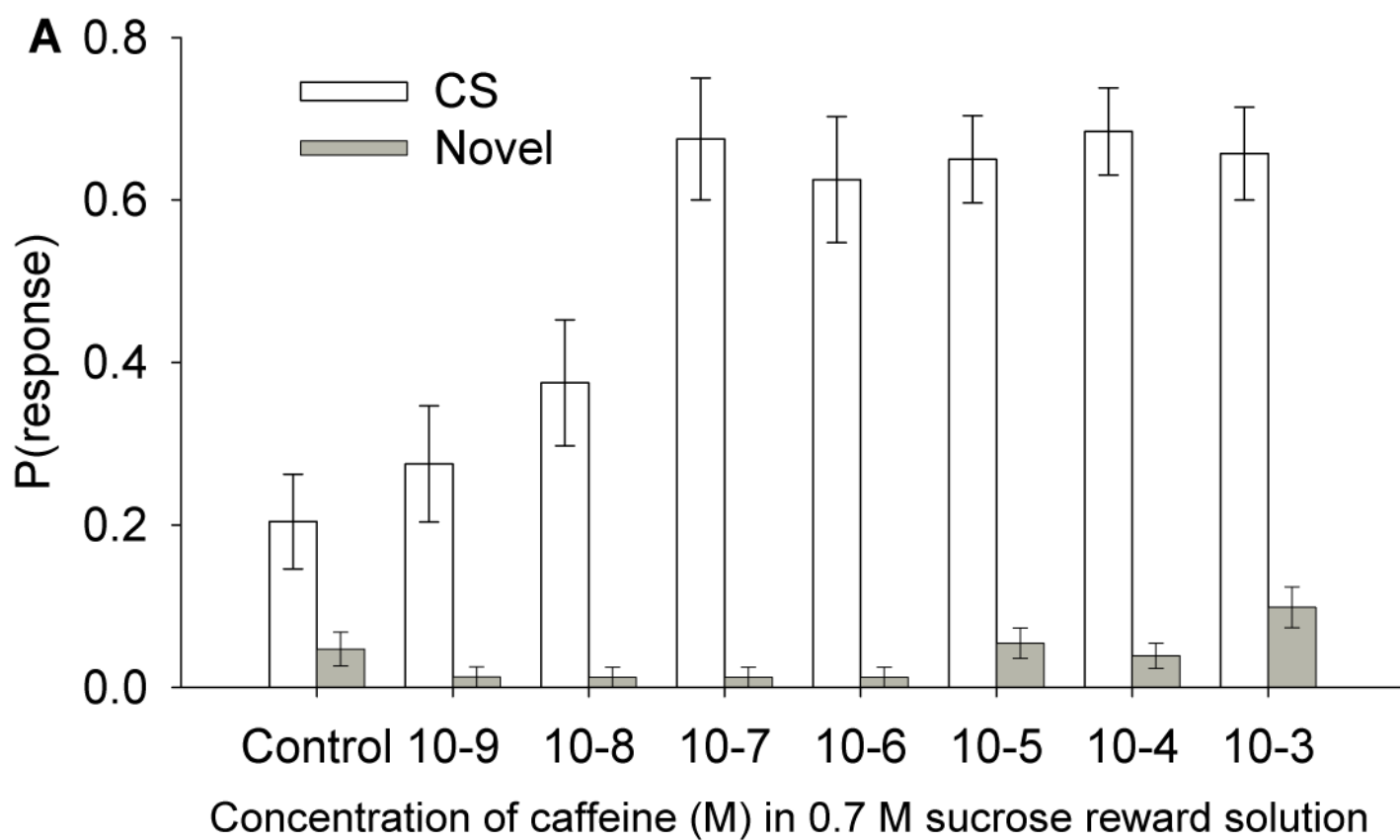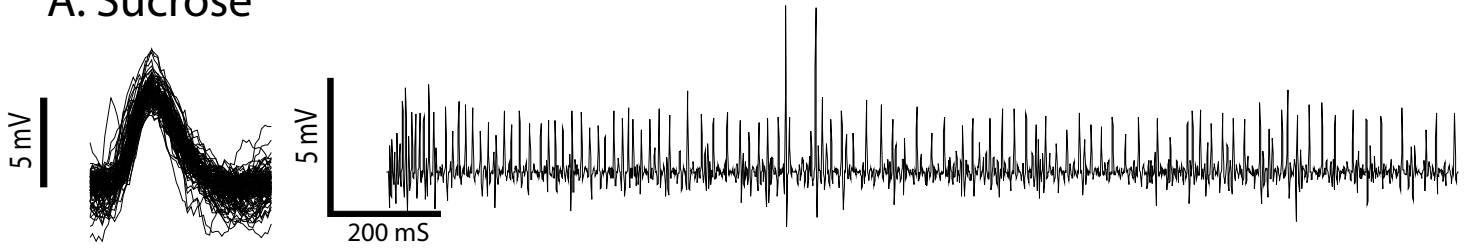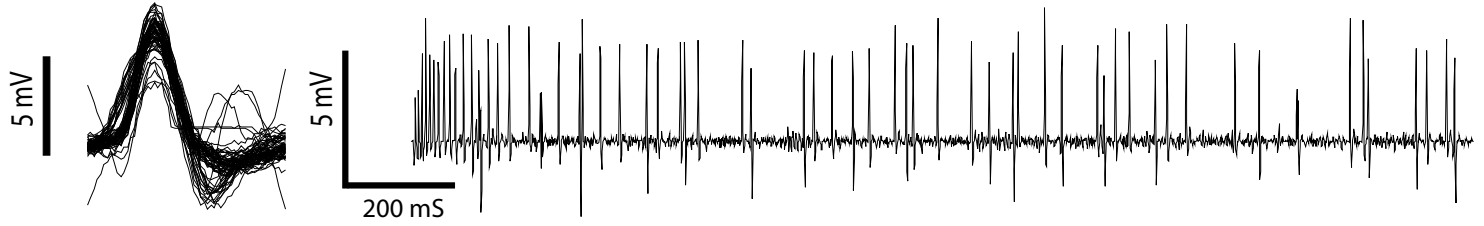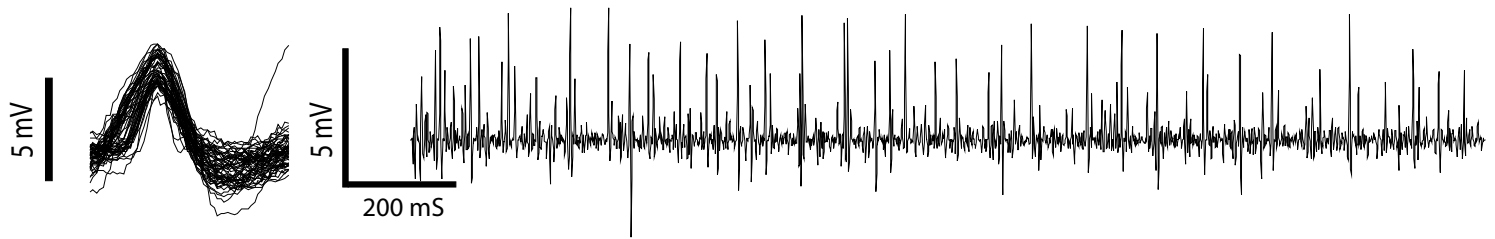Supplementary Materials present additional information in support of the conclusions of your paper, such as a description of the materials and methods, controls, or tabulated data presented in graphs or figures in the paper. It will generally consist of one integrated PDF file. Audio or movie files or large data tables can be presented as separate files. Further information is available here.

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Supplementary Materials for

Caffeine in floral nectar enhances a pollinator's memory of reward

G. A. Wright, D. D. Baker, M. J. Palmer, D. Stabler, J. A. Mustard, E. F. Power,

A. M. Borland, and P. C. Stevenson

Author whom correspondence should be addressed: jeri.wright@ncl.ac.uk

This PDF file includes:

Materials and Methods
SupplementaryText
Figs. S1 to S3

Materials and Methods

Nectar sampling and analysis. Nectar from *Coffea* sp. was collected from the coffee germplasm collection at CATIE (Centro Agronomico Tropical de Investigacion y Ensenanza), Turrialba, Costa Rica; nectar from *Citrus* was collected from orchards on the campus of TEI (Technological Education Institute), Heraklion, Crete. Flowers were protected from pollinators using mesh bags overnight; nectar was collected from each flower using a 35 µl capillary tube and stored at -20°C prior to analysis. Samples were diluted either 5 or 10 fold prior to caffeine analysis. LC–MS analysis of the nectar samples and coffee was carried out using a Waters Alliance LC solvent delivery system with a ZQ MS detector on a Phenomenex Luna C18(2) column (150 X 4.0 mm i.d., 5µm particle size) operating under gradient conditions, with A = MeOH, B = H$_2$O, C = 1% HCO$_2$H in MeCN; A = 0%, B = 90% at t = 0 min; A = 90%, B = 0% at t = 20 min; A = 90%, B = 0% at t = 30 min; A = 0%, B = 90% at t = 31 min; column temperature 30°C and flow rate of 0.5 ml min^{-1}. Caffeine was purchased commercially (Sigma-Aldrich, Dorset, UK) and used as a chromatographic standard and producing a calibration curve by quantification of the [M+H]$^{+}$ molecular ion with *m/z* = 195.1. Caffeine eluted at 5.14 min. For sugar analysis, samples were diluted 1000-2000 fold prior to analysis of carbohydrates in nanopure water. HPLC analysis of sugars was conducted by injecting 20 µl of sample via a Rheodyne valve onto a Carbopac PA-100 column (Dionex, Sunnyvale, California, USA). Sample components were eluted from the column isocratically using 100mM NaOH flowing at 1 ml/min. The chromatographic profile was recorded using pulsed amperometric detection (ED40 electrochemical detector, Dionex). Elution profiles were analysed using PeakNET software package (Dionex, Breda, Netherlands). Daily reference curves were obtained for glucose, fructose, sucrose, sorbitol and mannitol by injecting calibration standards with concentrations of 10 ppm for each sample.

Bee conditioning and taste assays. In the field, we measured that the average inter-visit interval to flowers of *Citrus paradisi* was 20.3 s (N = 92) and that the average time spent on each flower was 3.77 s (N = 81). The average sum of the time between each reward was ~24 s (sum of inter-visit interval and time on flower). In our conditioning experiments, we therefore chose to use a 30 s inter-trial interval (also used by (*33*) in studies of massed conditioning in bees; it should be noted here that previous studies have shown that massed conditioning is less likely to produce long-term memories in sucrose-rewarded bees than spaced conditioning (*33*)). Each subject was collected, restrained, and fed to satiety using an 'ad libitum' method with 0.7 M sucrose at 24 h prior to conditioning. In this method of feeding, honeybees are stimulated to extend the proboscis; the proboscis is placed in contact with sucrose solution in a 1 ml well and the bee allowed to drink until it reaches satiation. On the day of conditioning, each bee was tested for its motivation to feed by eliciting the proboscis extension response with 0.7M sucrose; bees that did not respond with a feeding reflex were deemed unmotivated and therefore not included. Each bee was trained by placing it in the conditioning arena, exposing it to a 4 s odour pulse with 1-hexanol (as described in (*34*)), and feeding it with a 0.4 µl droplet of 0.7M sucrose solution containing caffeine. Bees were trained for 6 trials; two unreinforced test trials (one with 1-hexanol and another with a novel odour, 2-

octanone) were conducted 10 min after the last conditioning trial and 24 h later. Bees were fed with 0.7 M sucrose ad libitum between the 10 min and 24 h test and left in a closed, humid box overnight. Data for conditioning and testing represent the probability of a response from the population on each trial. Sample sizes for each of the treatments are as follows: conditioning and 10 min test: $N_{control} = 156$, $N_{10-9M} = 79$, $N_{10-8M} = 80$, $N_{10-7M} = 80$, $N_{10-6M} = 80$, $N_{10-5M} = 159$, $N_{10-4M} = 160$, $N_{10-3M} = 160$; 24 h test: $N_{control} = 106$, $N_{10-9M} = 79$, $N_{10-8M} = 80$, $N_{10-7M} = 80$, $N_{10-6M} = 80$, $N_{10-5M} = 147$, $N_{10-4M} = 154$, $N_{10-3M} = 143$. (Differences in sample sizes from 10 min to 24 h were due to overnight mortality of subjects).The taste assay for mouthparts sensitivity to bitter compounds was conducted as described in (27). Determination of the taste threshold was performed by comparison against the sucrose-only control: we identified the threshold for the detection of caffeine by measuring when the drinking response towards the solution was significantly lower than that produced by sucrose alone.

Whole-cell patch-clamp electrophysiology: Adult worker honeybees (*Apis mellifera mellifera)* were anesthetized on ice and the intact brain isolated while submerged in extracellular solution. Surrounding tissue and membranes were removed by a combination of manual dissection and treatment for 5-10 min with papain (0.3 mg.ml^{-1}), L-cysteine (1 mg.ml^{-1}), collagenase (64 µg.ml^{-1}) and dispase (0.7 mg.ml^{-1}) (4). The removal of covering membranes was necessary to obtain successful whole-cell recordings from KCs. The brain was transferred to the recording chamber, secured with a mesh weight and continuously perfused with extracellular solution comprising (mM): NaCl (140), KCl (5.0), $MgCl_2$ (1.0), $CaCl_2$ (2.5), $NaHCO_3$ (4.0), NaH_2PO_4 (1.2), hepes (6.0), glucose (14), adjusted to pH 7.4 with NaOH, 326 mOsm, at room temperature (18-22 °C) (35-36). Caffeine and DPCPX were bath-applied via the extracellular solution. Acetylcholine (ACh) was pressure-applied using a Picospritzer from a glass micropipette positioned 25-50 µm from the recorded KC.

Whole-cell voltage-clamp and current-clamp recordings were obtained from visually-identified mushroom body KCs. Patch pipettes (8-10 MΩ) were pulled from borosilicate glass and filled with solution comprising (mM): K-gluconate (110), hepes (25), KCl (10), $MgCl_2$ (5), Mg-ATP (3), Na-GTP (0.5), EGTA (0.5), pH 7.2, 284 mOsm. Membrane current (I_M) and voltage (V_M) were recorded via an EPC-10 patch-clamp amplifier controlled by Patchmaster software (HEKA). Holding potentials (V_H) and measured V_M were corrected after the experiment for a liquid junction potential of +13 mV. Voltage-clamp recordings were made at a V_H of -73 mV. Series resistance (R_S), input resistance (R_I) and membrane capacitance (C_M) were calculated from a double-exponential fit of the capacitative current. Mean C_M and R_I of neurons used for the experiments were respectively 5.2 ± 0.7 pF and 1.42 ± 0.13 GΩ (n = 18). Recordings were not used if I_M or V_M changes were accompanied by changes in R_S. Off-line analysis was performed using IgorPro software (WaveMetrics). Pooled data are expressed as mean ± SEM; statistical significance was assessed using paired or unpaired Student's *t* tests as appropriate, with P < 0.05 considered significant (*).
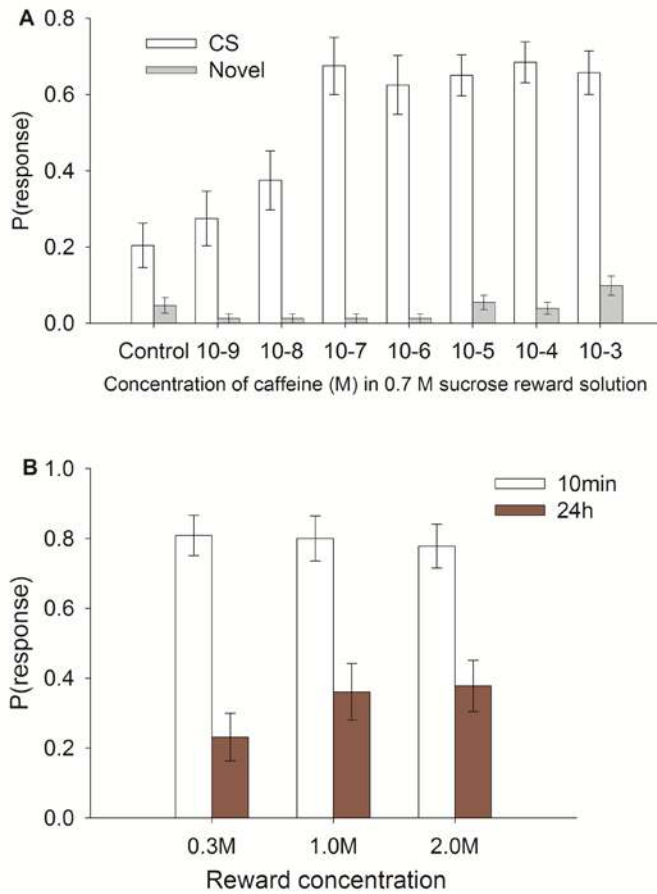
Tip recording electrophysiology. An electrophysiological recording was made from neurons located in a sensilla chaeticum located at the tip of the galea on the

honeybee's proboscis using the tip recording technique (*27*). All stimulating solutions contained 1 mM KCl as a conductive electrolyte. A glass electrode with a tip ~20 μm in diameter was used both for stimulating and recording. Recording commenced when the open end of the glass electrode was placed over the tip of the sensillum. It was connected to a TastePROBE amplifier (Syntech) and further amplified and filtered (CyberAmp 320, Axon Instruments; gain: 1000; eighth order Bessel pass-band filter: 1 Hz–2800 Hz). Each stimulus trial was digitised (sampling rate 10 kHz, 16 bits; DT9803 Data Translation), stored on a computer, and then analysed with the software, dbWave (http://taste.versailles.inra.fr/deterrents/tk/dbwave/).
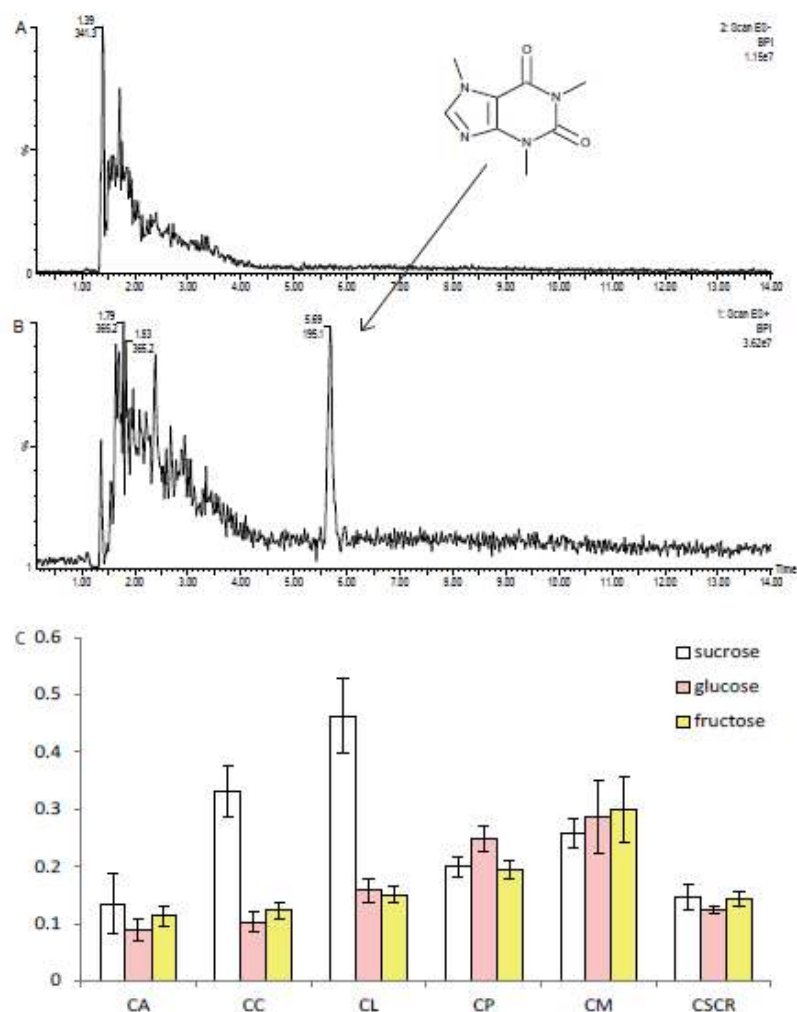
Fig. S1.

(**A-B**) Total Ion Chromatogram of *Coffea canephora* nectar using electrospray ionisation in both negative (**A**) and positive (**B**) mode and diluted 1:5 with distilled water. A 10 μl injection showing the well resolved single peak for caffeine in the nectar with inset mass spectrum extracted at 5.706 min characterised by a prononated molecular ion at *m/z* 195.1 $[M+H]^+$, as the major secondary metabolite detected in the nectar. The molecular ion was used to quantify concentrations in all nectar samples analysed against a calibration curve of an authentic standard of caffeine. The trailing peak between T = 1.5 – 4.00 min is typical of a column overloaded with sugars from nectar. (**B**) The relative concentrations of glucose, fructose, and sucrose in nectar depended on the species sampled (2-way ANOVA: $F_{10, 483} = 7.31$, $P < 0.001$). Sucrose was higher in concentration than glucose and fructose in nectar in *Coffea liberica* and *Coffea canephora* (lsc, P < 0.05), and glucose was greater in concentration than fructose and sucrose in *Citrus paradisi* (lsc, P < 0.05); in the other species, the concentration of glucose, fructose, and sucrose was not significantly different (lsc, P > 0.05). $N_{cc} = 34$, $N_{cl} = 31$, $N_{ca} = 27$, $N_{cp} = 17$, $N_{cm} = 5$, $Ncs = 7$, $N_{crcs} = 5$.

5

A 0.8

P(response)

0.6

0.4

0.2

0.0

CS
Novel

Control 10-9 10-8 10-7 10-6 10-5 10-4 10-3

Concentration of caffeine (M) in 0.7 M sucrose reward solution

B 1.0

P(response)

0.8

0.6

0.4

0.2

0.0

10min
24h

0.3M 1.0M 2.0M

Reward concentration

Fig. S2

(**A**) Caffeine consumed during acquisition did not increase the general olfactory responsiveness of bees during the 24 h test. Data represents the responses of honeybees conditioned and tested in Figures 2 and S2 to the conditioned odour (clear bars) and a novel odour (grey bars). The average response to the conditioned and novel odours depended on the concentration of caffeine (logistic regression, $\chi_7^2 = 14.6$, P = 0.040), but the responses elicited by the novel odour of the bees conditioned with caffeine was not greater than the responses of bees conditioned with 0.7 M sucrose (lsc, P > 0.10 for all comparisons). N > 80 for all groups. (**B**) Reward sucrose concentration does not influence long-term memory formation. Bees were massed-conditioned for 6 trials with an odour paired with 0.3 M, 1.0 M, or 2.0 M sucrose, and then tested with the odour at 10 min and 24 h after conditioning. At 24 h, honeybees were less likely to respond to the conditioned odour (logistic regression, $\chi_1^2 = 51.6$, P < 0.001). Reward concentration did not affect the response at either time during the test (logistic regression, $\chi_2^2 = 1.66$, P = 0.439). N > 40 for all 3 groups
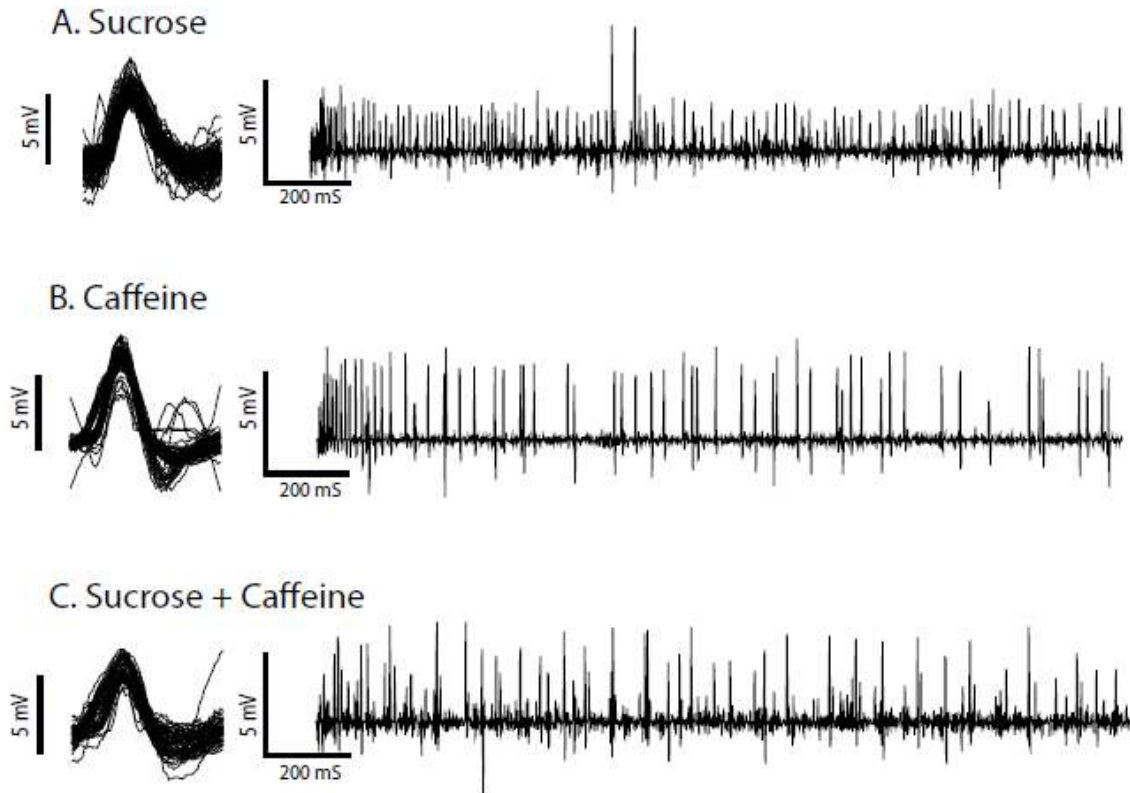
Fig. S3

Gustatory neurons in sensilla on the honeybee's mouthparts can detect caffeine in sucrose solution. A tip recording was made from the honeybee's galeal sensilla as reported in Wright *et al.* (2010) (*27*). Recordings were made for 2 s; traces are representations from the same sensillum. (**A**) The response to 0.3M; (**B**) the response to 0.01 M caffeine; (**C**) the response to a mixture of 0.3M sucrose and 0.01M caffeine.

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