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Journal of Chemical Ecology

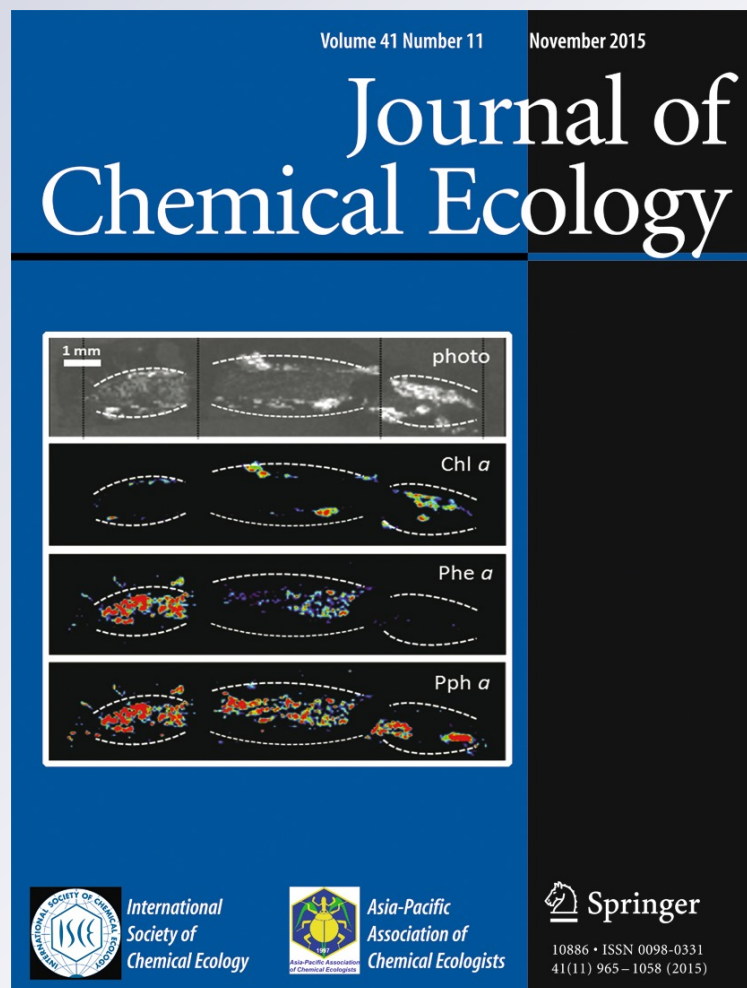
ISSN 0098-0331

Volume 41

Number 11

J Chem Ecol (2015) 41:1028-1036

DOI 10.1007/s10886-015-0642-2



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Nectar Attracts Foraging Honey Bees with Components of Their Queen Pheromones

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Received: 1 July 2015 / Revised: 8 October 2015 / Accepted: 15 October 2015 / Published online: 28 October 2015
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Abstract Floral nectar often contains chemicals that are deterrent to pollinators, presenting potential challenges to outcrossing plant species. Plants may be able to co-opt pollinator chemical signals to mitigate the negative effects of nectar deterrent compounds on pollination services. We found that buckwheat (*Fagopyrum esculentum*) and Mexican sunflower (*Tithonia diversifolia*) produce nectar with abundant phenolics, including three components of the *Apis* honeybee queen mandibular pheromone (QMP). In addition, these nectars contain a non-pheromonal phenolic, chlorogenic acid (CA), which was toxic to honeybees, and *T. diversifolia* nectar also contained isochlorogenic acid (IA). Fresh nectar or solutions containing nectar phenolics reduced *Apis* individual feeding compared to sucrose solutions. However, freely foraging bees preferred solutions with QMP components to control solutions, and QMP components over-rode or reversed avoidance of CA and IA. Furthermore, prior exposure to the presence or just the odor of QMP components removed the deterrent effects of CA and IA. By mimicking the honey bee

pheromone blend, nectar may maintain pollinator attraction in spite of deterrent nectar compounds.

Keywords Bee mandibular pheromone · Chemical mimicry · Nectar · Phenolics · Pollination

Introduction

The challenges of attracting mates while avoiding predation are well recognized in animal systems. Plants, as sessile organisms, are particularly reliant on chemical traits to mediate interactions, both with antagonists and with pollinators that can be essential for reproduction. While extensive research has examined how pollinators perceive flowers and make foraging choices, chemical interactions between flowering plants and pollinators can be complex (Dötterl and Vereecken 2010). The evolution of insect perceptual systems predated the evolution of floral color and scent in angiosperms (Briscoe and Chittka 2001; Schiestl 2010), providing the opportunity for plant signals to evolve in part by exploiting pre-existing sensory biases in pollinators (Schiestl et al. 2010). In some well-known examples of floral sexual mimicry, orchid flowers produce both visual and volatile cues that mimic female insects, thus encouraging male insects to display copulating behavior that also transfers pollen (Vereecken and Schiestl 2008). However, there is broader potential for the evolution of floral traits that take advantage of pre-existing insect preferences regardless of coevolutionary history. A recent review found 87 % overlap between the volatile organic compounds produced by flowers and insects, providing widespread support for the hypothesis of overlapping signals between these interacting taxa (Schiestl 2010).

In addition to producing attractive visual and volatile signals, many plant species contain deterrent compounds in

Electronic supplementary material The online version of this article (doi:10.1007/s10886-015-0642-2) contains supplementary material, which is available to authorized users.

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nectar and pollen (Adler 2000; Heil 2011). Such compounds may have evolved to deter nectar robbers, preserve nectar against spoilage, or encourage specialized pollinators or more efficient pollinator behavior (Adler 2000; Heil 2011). Alternatively, these compounds could be a pleiotropic consequence of defense production in other plant tissues (Adler et al. 2012). Regardless of their evolutionary origins, many nectar compounds are deterrent to pollinators (Adler 2000; Heil 2011), which may represent a challenge to outcrossing species that depend on pollinators for reproduction (Adler and Irwin 2005; Adler et al. 2012; Galen et al. 2011; Kessler and Baldwin 2007). The presence of floral chemicals that co-opt insect signals may allow the persistence of deterrent compounds without sacrificing pollinator attraction.

Here, we focused on two distantly related plant species, one native and one introduced, that occur commonly in China and are often visited by honey bees as pollen and nectar sources. One is the common buckwheat, *Fagopyrum esculentum* (Polygonaceae), which is a widely planted distylous crop that strictly requires insects to provide cross-pollination between the 'pin' and 'thrum' morphs (Cawoy et al. 2008). In the mountains of Yunnan, China, which is a center of origin of *F. esculentum* (Ohnishi 1991), the native honey bee *Apis cerana* (Ruttner 1988) is a legitimate pollinator of *F. esculentum* (Björkman 1995). Our other focal plant species was the Mexican sunflower, *Tithonia diversifolia* (Asteraceae), which is an invasive shrub in tropical Asia (Sharrock et al. 2004). It is pollinated partly by insects (Mukhopadhyay et al. 2007), including *A. cerana* (Mukhopadhyay et al. 2007) and *A. mellifera* (Kayode and Oyeyemi 2014). We demonstrated that both species produce nectar containing components of the *Apis* queen mandibular gland secretion, usually termed queen mandibular pheromone (QMP), and that exposure to these nectar components altered *Apis* bee feeding responses to nectar deterrents at concentrations found in nectar. Our results reveal for the first time that nectar can contain components of bee pheromones that remove the deterrent effects of nectar compounds on bee pollinators.

Methods and Materials

Analysis of Sugars and Phenolics in Nectar Nectar was extracted from 15 plants of each floral morph of *F. esculentum* from a farm in Mile county (103°25'E, 24°24' N, elevation 1050 m) of Yunnan province on August 1, 2010, and 9 plants of *T. diversifolia* in the field in Menghai county (99°58'E, 21°55'N, elevation 535 m) of Yunnan province on December 5, 2008 using 1 μ l glass micropipettes (Hirschmann Laborgeräte, Eberstadt, Germany). Nectar was pooled across several plants of each morph of *F. esculentum* or across flowers within individual *T. diversifolia* plants to obtain

sufficient volumes (50 μ l) for samples. For the analysis of nectar sugar concentration, three 1 μ l samples were measured with a low-volume hand refractometer (Atago HSR 500), and the sugar concentration was expressed as a percentage of sucrose in the total nectar mass (wt/wt).

For analysis of nectar phenolics, three nectar samples (50 μ l/each) of *T. diversifolia* or each morph of *F. esculentum* were frozen immediately in liquid nitrogen and stored at -80°C until analysis. High performance liquid chromatography (HPLC) was used to determine phenolic composition and concentrations of samples (Liang et al. 2009). An Agilent 1100 liquid chromatography system (Agilent Technologies Deutschland, Waldbronn, Germany), equipped with a vacuum degasser, a quaternary solvent delivery pump, a manual chromatographic valve, a thermostated column compartment, a diode-array detector (DAD; Agilent, Palo Alto, CA, USA), and a HP1049A programmable electrochemical detector (ECD; Hewlett Packard, USA) was used. In brief, each sample was extracted with 0.5 ml of methanol at 25°C for 1 h, sonicated for 15 min, filtered, and 10 μ l were analyzed by HPLC with a Zorbax SB-C18 column (150 mm \times 4.6 mm, 5.0 μ m); temperature = 30°C ; solvent A- 2 % (v/v) aqueous formic acid, solvent B- methanol; flow rate: 1.0 ml min^{-1} ; Gradient: 0–6 min- 2 % B, 6–10 min- 8 % B, 10–18 min- 15 % B, 18–23 min- 35 % B, 23–28 min- 55 % B, 28–33 min- 70 % B; electrochemical detector (Agilent 1049A), 0.8 V, oxidative mode. This is an HPLC-DAD-ECD system, in which the mobile phase first enters the DAD, which provides UV spectra and retention times that are used for compound identification, and then to the ECD, which provides a more sensitive electrochemical detection that was used for quantification. Compounds were identified by comparison of retention times and UV spectra to those of commercially available reference chemicals (Sigma-Aldrich) following the protocol developed by Liang et al. (2009). Quantification was carried out using standard curves produced with reference compounds.

General Bioassay Procedures All assays were conducted at the Xishuangbanna Tropical Botanical Garden (101°25'E, 21°41'N, elevation 570 m), Chinese Academy of Sciences. Sucrose solutions with or without phenolics were prepared fresh for each test. Mixtures of 4-hydroxy-3-methoxyphenylethanol (HVA), 4-hydroxybenzoic acid (HB), and ferulic acid (FA) are referred to as the "QMP blend" or "QMP" for brevity. The non-pheromonal phenolics we examined were chlorogenic acid (CA) and isochlorogenic acid (IA); the latter was found in *T. diversifolia* nectar only. Compounds were obtained from Sigma-Aldrich (chlorogenic acid, $\geq 95\%$ purity, ferulic acid, $\geq 98\%$), Shanghai Jianchao (4-hydroxy-3-methoxyphenylethanol, $\geq 98\%$), Shanghai Haoran (4-hydroxybenzoic acid, $\geq 98\%$), and Aladdin (isochlorogenic acid, $\geq 98\%$). For all bioassays with *A. cerana*, the sucrose

concentration was 50 % (w/w), and the contents of HVA, HB, FA, and CA were 0.3, 2.0, 0.1, and 1.5 mg/100 g of solution, respectively, similar to the concentrations found in *F. esculentum* nectar (see insert, Fig. 1a). For all bioassays using *A. mellifera*, the sucrose concentration was 30 % (wt/wt), and the contents of HVA, HB, FA, CA, and IA were 0.05, 0.5, 0.03, 2.0, and 1.5 mg/100 g of solution, matching the concentrations found in *T. diversifolia* nectar (see insert, Fig. 1b). The colonies of the two bee species came from local beekeepers, and all experiments were conducted in an outdoor flight cage near the laboratory.

Effect of Nectar Phenolics on Individual Feeding To determine whether nectar phenolics reduce nectar consumption by individual *A. cerana* bees, we conducted feeding assays with individual harnessed bees in our laboratory (25 °C and relative humidity 70 %) in November and December 2012. Bees were captured at the hive entrance of one healthy colony with a queen for all bioassays. They were anesthetized in a bottle bathed in ice water and harnessed in tubes. After recovering, they were offered 50 % sucrose solution *ad libitum*, and were kept in a black box that was placed in a temperature-controlled chamber at 25 °C with 60 % relative humidity. Two h later, we offered individual bees one of five solutions with a micropipette: pure sucrose solution, the sucrose solution with CA, the sucrose solution with QMP components, the sucrose solution with QMP components plus CA, and the nectar of *F. esculentum*. To avoid feeding-time bias, we offered the first bee pure sucrose solution, the second the CA solution, the third the QMP component solution, the fourth the QMP components plus CA, and the fifth nectar. Then we reversed the feeding order for the next replicate set of bees. Each bee was offered their treatment solution for 20 min. Bees were the unit of replication, and a total of 40 bees were offered each treatment for each trial. The response variable was the weight of solution consumed, calculated by weighing the bees before and after feeding. We used the Pauta criterion to eliminate outliers and achieve a normal distribution, and then compared individuals' intake of different solutions by using a Tukey's studentized range test with 20-min consumption (mg/bee) as the response, and solution treatment (sucrose, sucrose+CA, sucrose+QMP, sucrose+CA+QMP, or nectar) as the explanatory factor. We used the same methods to examine the feeding response of individual *A. mellifera* to *T. diversifolia* nectar or its components, replacing the CA treatments with CA and IA since both phenolics are found in this species.

Effect of Nectar Phenolics on Colony Foraging To determine whether nectar phenolics affect bee feeding choices at the colony level, we conducted binary feeding choice experiments using *A. cerana* colonies and solutions with phenolic compositions similar to *F. esculentum* nectar. In August 2011, we moved a food-deprived colony (~4000 workers with a

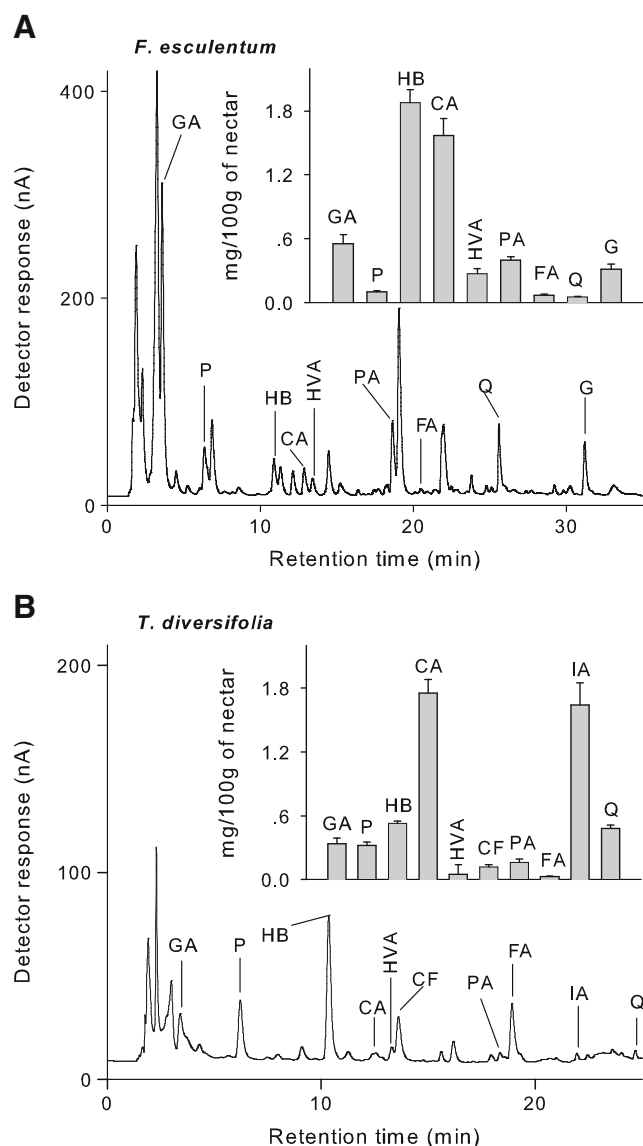


Fig. 1 Abundant phenolics are present in the nectar of two distantly related plant species. Phenolic composition of (a) *Fagopyrum esculentum* nectar or (b) *Tithonia diversifolia* nectar is depicted by an HPLC chromatogram and a graph of individual quantities (insert). Gallic acid (GA), Protocatechuic acid (P), 4-hydroxybenzoic acid (HB), Chlorogenic acid (CA), 4-hydroxy-3-methoxyphenylethanol (HVA), caffeic acid (CF), *p*-coumaric acid (PA), ferulic acid (FA), isochlorogenic acid (IA), quercetin (Q), and galangin (G). Bars represent means±standard errors. $N=6$ samples for *F. esculentum* (3 of each morph) and 3 for *T. diversifolia*

single queen) into a flight cage (25×10×5 m), and trained its bees to visit two feeders containing 30 % sucrose solution, which were 50 cm apart and 5 m away from the hive. When bees regularly visited the training feeders, we marked the abdomens of the next 20 foragers that fed on the solution (Edding 751 marker paint, Germany) to make sure we did not collect these bees for the experimental assay, and released them. To simulate *F. esculentum* nectar, we used 50 % sucrose in two experimental feeders, one with and the other without

CA. We captured newly recruited bees (unmarked) at each experimental feeder for a total of 90 min, and assessed recruitment as the percent of total bees captured at each feeder. Because all bees recruited to the treatment feeders were captured, there was no opportunity for bees to return to the colony and communicate with nestmates. Thus, each bee that visited a feeder was naïve to the treatment solutions, and bees in the hives were not influenced by the choices of their nestmates, precluding the possibility that bees learned to associate scent with reward. Each experiment was replicated with three different colonies on separate dates (Aug 1, 2, and 6) with at least 150 bees captured for each trial; trials were the unit of replication ($N=3$). A G -test was used to test whether the mean of the observed frequencies of bee recruits to two solutions deviated significantly from chance (*i.e.*, 50 % of total captured bees recruited to each feeder). Using the same methods, we also examined bee feeding choices between the pure sucrose solution and the same sucrose solution with the QMP components found in *F. esculentum* nectar, as well as between the sucrose solution with QMP components and the solution with QMP components plus CA.

We conducted parallel feeding choice experiments with *A. mellifera* colonies in August 2010. After foragers regularly visited training feeders with a 30 % sucrose solution, we presented colonies with two experimental feeders containing one of the following pair of treatments: sucrose solution *vs.* sucrose solution with CA and IA, sucrose solution *vs.* sucrose solution with QMP components, or sucrose solution with QMP components *vs.* the solution with QMP components plus CA and IA. The concentration of sucrose solution was always 30 % (w/w), like *T. diversifolia* nectar. Three colonies were tested for each of the three pairs of treatments on separate dates (Aug 18, 20, and 21 for the first colony; Aug 23, 24, and 27 for the second colony, and Aug 29, 30, and 31 for the third colony). G -tests were used to test whether the mean of the observed frequencies of bee recruits to two solutions of each pair deviated significantly from chance.

Effect of Exposure to QMP on Apis Preference To test whether exposure to QMP components altered bee feeding preference for nectar deterrents, we conducted related experiments in *A. cerana* and *A. mellifera*. In all experiments, we manipulated exposure to QMP components and then offered bees sucrose solutions with or without deterrent phenolics, always in the absence of QMP components. Thus, we eliminated the possibility of associative learning or innate preferences for odors, and isolated the effect of pre-exposure to QMP components on subsequent response to deterrent phenolics.

For *A. mellifera*, we manipulated food provided inside replicate colonies. We replaced food and brood frames with empty ones for two colonies (each with ~4000 workers), and then moved them into the flight cage as above and trained bees to

visit feeders 5 m away from the hives. At dusk, we provided a feeder inside each hive that contained 200 ml of 30 % sucrose solution or the same sucrose solution with QMP components. The next day we compared freely flying bees' recruitment to the two feeders, which contained a sucrose solution or a sucrose solution with CA and IA. The experiment was replicated three times, with colony as the unit of replication. G -tests were used to test whether the mean of the observed frequencies of bee recruits from sucrose-fed or QMP-fed colonies to the two solutions of each pair deviated significantly from chance.

We further examined whether exposure to the odor of QMP components alone was sufficient to induce a change in individual feeding preferences in *A. cerana*. Foraging *A. cerana* bees were captured and harnessed in the same way as for the individual feeding trials above. After recovering, we offered them 50 % sucrose solution *ad libitum*, and exposed them to 10 ml of either 50 % sucrose solution or the solution with QMP components in a Petri dish inside a black box. Because bees were harnessed, those in the QMP treatment were exposed to the odor of QMP components but could not consume it. The box was kept in a temperature-controlled chamber at 25 °C with 60 % relative humidity. Two h later, we offered individual bees from each odor treatment 50 % sucrose solution or the solution containing CA for 20 min. To avoid feeding-time bias, we first offered a sucrose-exposed bee the pure solution, followed by a sucrose-exposed bee offered the CA-laced solution, a QMP-exposed bee offered pure solution, and a QMP-exposed bee offered the CA-laced solution. After that, we reversed the feeding order for the next replicate. A total of 40 bees were used for each treatment. We calculated total individual solution intake by weighing bees before and after feeding. We used the Pauta criterion to eliminate outliers and achieve a normal distribution, and then compared individual intake of bees in each treatment using Tukey's studentized range test.

Results

Identification of the QMP Components in Nectar We detected nine phenolics in *F. esculentum* nectar, including 4-hydroxybenzoic acid (HB) and chlorogenic acid (CA), making up 36.1 % and 30.2 %, respectively, of the total phenolic composition (Fig. 1a and insert). This nectar also contained two minor phenolics, 4-hydroxy-3-methoxyphenylethanol (HVA) and ferulic acid (FA) (inserts, Fig. 1a). 4-Hydroxy-3-methoxyphenylethanol is a major component of *A. mellifera* QMP (Plettner et al. 1997), while HB and FA are minor components of *A. cerana* QMP (Keeling et al. 2001).

In *T. diversifolia* nectar we detected ten phenolics (Fig. 1b), including CA and isochlorogenic acid (IA), which made up 30.8 % and 28.9 %, respectively, of the total phenolic composition (Fig. 1b and insert). We also detected HVA, HB, and

FA, the QMP components, as minor phenolics (Fig. 1b and insert). This is the first time that components of *Apis* queen pheromones have been detected in nectar. We refer to these compounds as ‘QMP components’ hereafter, while recognizing they were produced by the plant rather than insects. It is notable that these components were found in two distantly related plant species, suggesting that broader surveys of nectar phenolics would be valuable to determine the generality of this phenomenon.

Effect of Nectar Phenolics on Individual Feeding CA and IA have been reported to deter herbivores such as thrips (Leiss et al. 2009). We found that concentrations of approximately 4 mg/100 g killed half the *A. cerana* bees in 24 hr. By comparison, natural nectar concentrations were 1.5 mg/100 g in *F. esculentum* and 2.0 mg/100 g in *T. diversifolia*, indicating that nectar of both plant species has sublethal concentrations of CA (see Online Resource 1). We then measured consumption of sucrose solutions with and without CA offered to individual harnessed *A. cerana* bees. Compared to the pure sucrose solution, bees consumed similar amounts of the solution with CA (Tukey’s studentized range test, $P=0.313$) and the solution with QMP components ($P=0.145$), but were deterred by solutions with QMP plus CA ($P<0.001$) and fresh *F. esculentum* nectar ($P<0.001$; Fig. 2a). Thus, the combination of CA and QMP components reduced *A. cerana* feeding compared to a sucrose control solution, and *F. esculentum* nectar, which includes CA and QMP compounds, was unpalatable to *A. cerana* foragers.

Similarly, harnessed individual bees of *A. mellifera* drank more of the pure sucrose solution than the solution with CA and IA (Tukey’s studentized range test, $P=0.035$; Fig. 2b). Although sucrose solutions with the QMP compounds or the QMP blend plus CA and IA did not reduce feeding compared to the control solution ($P=0.676$ and 0.322 , respectively; Fig. 2b), *T. diversifolia* nectar significantly reduced feeding ($P=0.001$; Fig. 2b).

Effect of Nectar Phenolics on Colony Foraging We conducted feeding choice experiments with *A. cerana* colonies (Fig. 3a). When an *A. cerana* colony was given a choice between a sucrose solution and the sucrose solution with CA, fewer bees were recruited to the sucrose solution with CA ($G=7.545$, $P=0.006$). By contrast, when an *A. cerana* colony was given a choice between a sucrose solution and the sucrose solution with the QMP components, more bees were recruited to the sucrose solution with the QMP components ($G=9.313$, $P=0.002$). However, recruitment to the QMP components plus CA was not different from recruitment to the sucrose solution with QMP components ($G=0.180$, $P=0.671$).

We found similar results using *A. mellifera* colonies (Fig. 3b). Fewer bees were recruited to solutions with CA and IA compared to the sucrose control ($G=10.279$, $P=$

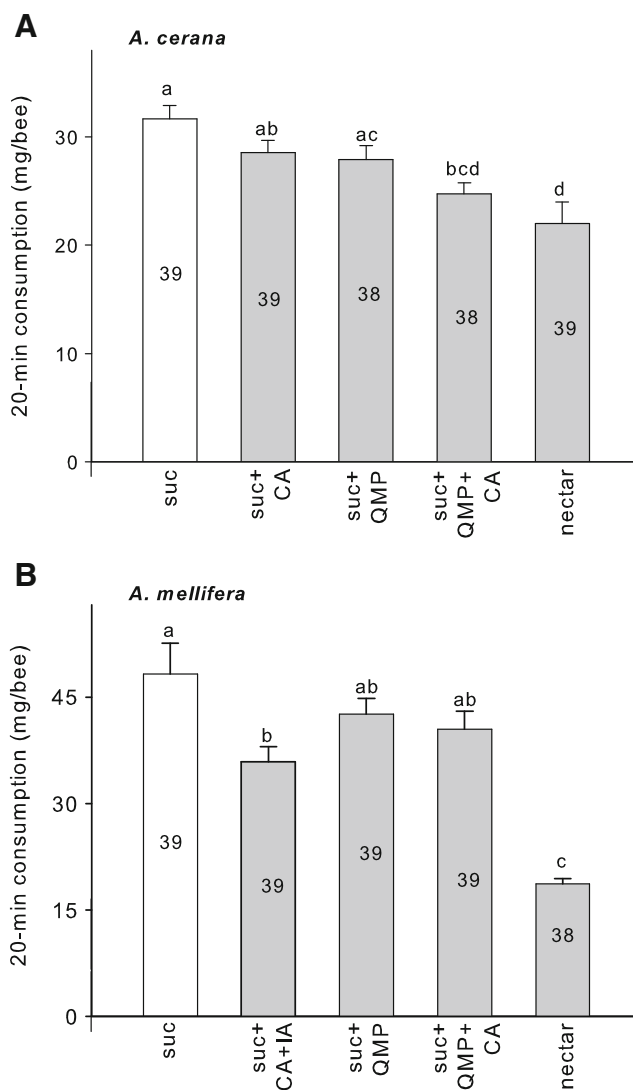


Fig. 2 Individual feeding by restrained bees was reduced by phenolics, including queen mandibular pheromone (QMP) components. (a) Individual feeding by *Apis cerana* with *Fagopyrum esculentum* nectar or solutions containing phenolic components. (b) Individual feeding by *A. mellifera* with *Tithonia diversifolia* nectar or solutions containing phenolic components. Sucrose solution (suc), QMP, a blend of 4-hydroxy-3-methoxyphenylethanol (HVA), 4-hydroxybenzoic acid (HB), and ferulic acid (FA); nectar, the nectar of *F. esculentum* or *T. diversifolia*. The concentrations of sucrose and phenolics were those detected in *F. esculentum* or *T. diversifolia* nectar. Forty bees were offered each solution. Arabic numerals in bars denote the numbers of bees after excluding outliers. Bars represent means±standard error. Means followed by the different letters are significantly different ($P=0.05$, Tukey’s studentized range test)

0.001), but more bees were recruited to solutions with QMP components compared to the sucrose controls ($G=17.356$, $P<0.001$). Furthermore, more bees were recruited to solutions with QMP compounds plus CA and IA than to the sucrose solution with QMP components only ($G=5.986$, $P=0.014$).

Thus, CA and IA were deterrent to both individual bee and colony feeding responses, but *A. cerana* foraging colonies were attracted to solutions with QMP components or

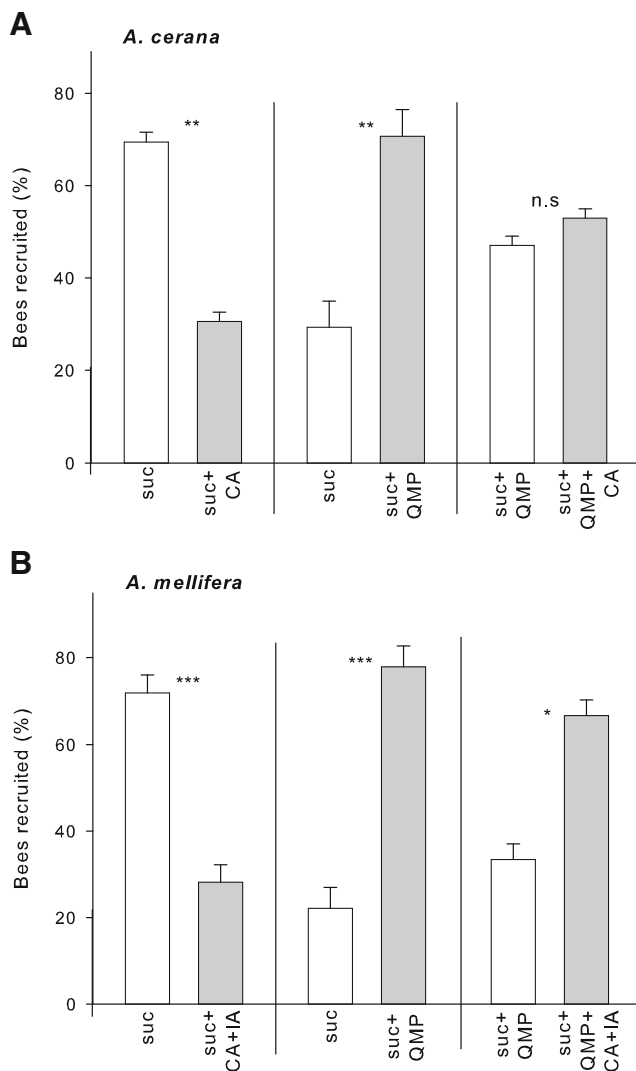


Fig. 3 Foraging bees recruited from honeybee colonies were deterred by the phenolics chlorogenic acid (CA) and isochlorogenic acid (IA), but attracted by their queen mandibular pheromone compounds. **(a)** Feeding responses of *Apis cerana* colonies to phenolics detected in *Fagopyrum esculentum* nectar. **(b)** Feeding responses of *A. mellifera* colonies to phenolics found in *Tithonia diversifolia* nectar. Y-axis depicts percent of bees recruited to each solution within trials. Sucrose solution (suc), CA, IA, queen mandibular pheromone (QMP), a blend of 4-hydroxy-3-methoxyphenylethanol (HVA), 4-hydroxybenzoic acid (HB), and ferulic acid (FA). Bees were captured after they chose a feeder, so that each foraging decision was a separate and independent event. Each experiment included three replicates (trials) with separate bee colonies; trial was the unit of replication and at least 150 separate foraging bees were assessed per trial. Bars represent means \pm standard error. Significance determined by *G*-tests: n.s. $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

QMP plus CA. Similarly, the presence of QMP components reversed the effects of CA and IA on *A. mellifera* feeding choices.

Effect of Exposure to QMP on Apis Preference To determine whether exposure to QMP altered responses to CA and

IA in *A. mellifera*, we fed *A. mellifera* bee colonies sucrose solutions inside their hives with or without the QMP compounds, and examined their feeding choice at feeders outside their hives between sucrose solutions and the same solution with CA and IA (Fig. 4a). Bees from the colonies fed sucrose avoided the solution with CA and IA ($G=4.632$, $P=0.032$), but those from the colonies fed QMP components preferred the solution with CA and IA ($G=5.280$, $P=0.022$). Thus, exposure to QMP components reversed bee responses to CA and IA.

Altered *A. mellifera* preference for CA and IA following QMP exposure could have been due to effects of consuming QMP, or of perceiving QMP *via* taste or olfaction that modified subsequent preferences. We further examined whether exposure to the odor of QMP components alone was sufficient to alter bee feeding responses to nectar deterrents with individual *A. cerana*. We harnessed individual foragers and placed them in a black box containing a sucrose solution or the solution with QMP, so that the bees could detect but not consume the solutions. We then offered the harnessed bees sucrose solutions with or without CA. Exposure to QMP component odors did not affect bees' feeding on pure sucrose solutions (comparison of sucrose-exposed and QMP-exposed bees to sucrose solutions; Tukey's studentized range test, $P=0.799$; Fig. 4b), thus indicating that QMP exposure did not reduce feeding responses to sugars. Bees exposed to a sucrose solution control were not deterred by CA, similar to the original experiment (comparison of sucrose-exposed bees offered sucrose vs. CA solutions; $P=0.852$; Fig. 4b). However, exposure to QMP component odors resulted in a preference for CA over the pure sucrose solution (comparison of sucrose-exposed bees and QMP-exposed bees offered CA solution; $P=0.026$; Fig. 4b). Thus, the odor of the QMP components did not significantly shift foraging bee feeding responses to sucrose, but greatly altered their responses to nectar deterrents.

Discussion

Our study showed that nectars of two distantly related plant species both contained the phenolic CA, which was toxic to bees. *Tithonia diversifolia* also contained the related phenolic IA, which was deterrent to both bee species. The presence of toxic compounds in nectar could have arisen prior to the plants' association with these pollinator species, and may have other benefits such as protecting flowers against visits by various antagonists (Adler 2000; Heil 2011), or protection against pathogen infection (McArt et al. 2014). Although many studies have speculated about the adaptive benefits of nectar secondary compounds to plants (Wright et al. 2013), few studies currently have assessed such benefits. Three found that nectar secondary compounds could promote pollen receipt

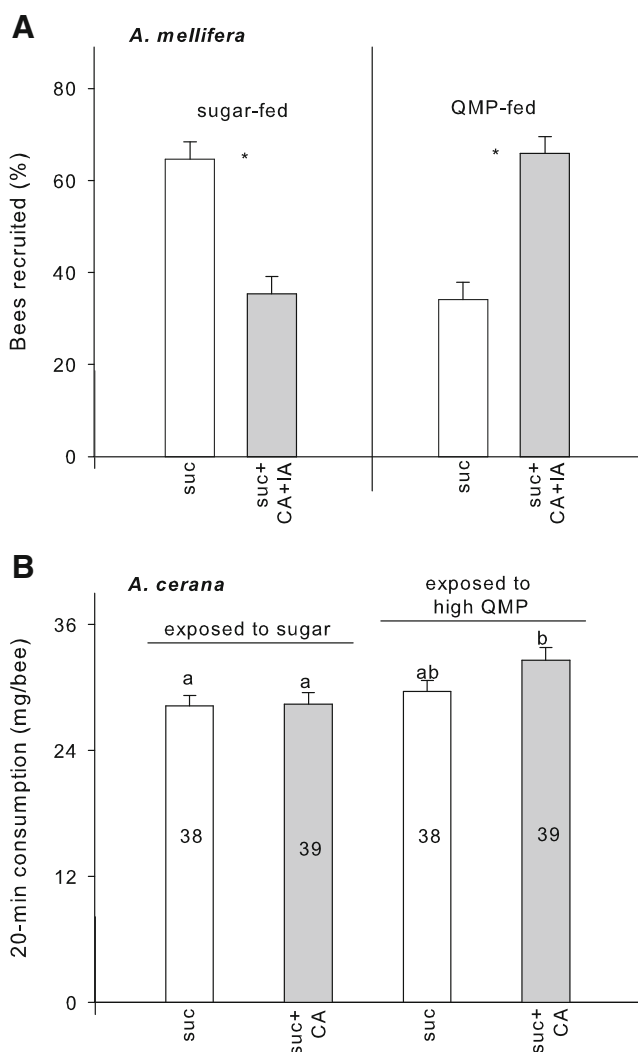


Fig. 4 Exposure to nectar queen mandibular pheromone (QMP) components fundamentally alters feeding responses. **(a)** Effect of QMP components or sucrose fed to *Apis mellifera* colonies on the percent of foraging bees that subsequently chose feeders with a sucrose control or sucrose with chlorogenic acid (CA) and isochlorogenic acid (IA). The experiment was repeated three times, using one pair of colonies for each trial. **(b)** Effect of exposure to QMP components or sucrose control on subsequent feeding rates of individual harnessed *A. cerana* foragers offered a sucrose control or sucrose with CA. Arabic numerals in bars indicate the numbers of bees after excluding outliers. Means followed by the different letters are significantly different ($P=0.05$, Tukey's studentized range test). Bars represent means \pm standard errors

(Thomson et al. 2015) and outcrossing (Kessler et al. 2008; Zhao et al. 2014), while one found that such compounds reduced pollen receipt and transfer (Adler and Irwin 2005). Furthermore, none of the compounds in those studies was toxic to bees at natural nectar concentrations (e.g., Elliott et al. 2008; Tiedeken et al. 2014), in contrast to the phenolics in the current study. Clearly more work is needed to assess the fitness costs or benefits of nectar compounds toxic to pollinators, and to understand the physiological and evolutionary origin of such traits.

Although the presence of toxic nectar compounds is interesting, our most novel results are the discovery of three components of *Apis* QMP in nectar, and the finding that exposure to these compounds fundamentally alters bee feeding responses to toxic phenolics. This is consistent with findings in several other mutualisms, in which components of nectar or similar secretions have manipulated partner behavior to the host's advantage (Heil et al. 2014; Hojo et al. 2015; Wright et al. 2013). It is notable that we found HVA, HB, and FA in the nectar of both plant species, while *A. mellifera* only produces HVA (Plettner et al. 1997), and *A. cerana* only produces HB and FA (Keeling et al. 2001). However, the combination of all three compounds had similar effects on both bee species. It is possible that only a subset of compounds would produce the effect in each species, or that the combination of all three is effective despite containing a novel component for each species. Thus, the mixture of these compounds is biologically significant in changing bee feeding behavior that may have consequences for plant fitness, even if these compounds are not all produced by both bee species. Our foraging trials (Fig. 3), compared the choices that free-flying bee choices made between solutions with or without toxic phenolics (Fig. 3a, b; far left panel) and with or without QMP components (Fig. 3a, b, far right panel). When both solutions contained QMP components, bees either did not discriminate between solutions with and without toxic phenolics (Fig. 3a), or they preferred solutions with toxic phenolics (Fig. 3b), indicating a fundamental shift in foraging choices. This change cannot be due to associating QMP components with toxic compounds, because QMP components were absent or present in both choices for those trials. More compelling, our final experiments showed that prior consumption of (Fig. 4a) or even exposure to the odor of QMP (Fig. 4b) changed bee feeding behavior to reverse or remove deterrent properties of toxic phenolics. Similar to our results, plant volatiles may induce a change in taste perception in herbivores (Matsuo et al. 2007). It has been shown that exposure to QMP can alter bee brain dopamine levels and dopamine receptors (Beggs et al. 2007). It is important to note that our experiments tested how QMP affected responses to toxic compounds, rather than the ability to learn to associate odors with toxins. Removing or reversing deterrence to toxins is presumably maladaptive for bees by allowing them to consume more toxins through foraging, but bees may be constrained in their ability to respond adaptively by the importance of QMP in critical hive functions (Liu et al. 2005). From the plant's perspective, QMP components in nectar could benefit plants by negating potential costs, in terms of pollinator deterrence, of toxic nectar phenolics that are present for other functions. Alternatively, perhaps the combination of QMP components and toxic phenolics reduces nectar consumption and increases delivery of outcross pollen, as has been found for nicotine in *Nicotiana attenuata* (Kessler et al. 2008). The fitness consequences of toxic phenolics and

QMP components for both the plants that produce them and bees that consume them merits future investigation.

Interactions between insect pheromones and plant secondary chemicals are well-known from other systems (Reddy and Guerrero 2004). For example, the herbivore defense compounds pyrrolizidine alkaloids are precursors to insect pheromones for some butterfly species (Hartmann et al. 2003; Schulz 1998), indicating that insects can respond to some plant defenses as potential pheromones rather than as deterrents. The pollen phenolic acid *p*-coumaric acid, a common component of bee bread fed to *A. mellifera* larvae, regulates genes involved in caste determination (Mao et al. 2015). Furthermore, studies in other systems have demonstrated that plant compounds can alter response to insect pheromones. For example, male moths of many species must rely on olfaction to find mates *via* perception of female pheromones, and to find food through processing information from plant volatiles (Chaffiol et al. 2014). In a wide range of systems, the addition of plant compounds can have synergistic effects that often increase, but sometimes decrease, aggregation responses to pheromones (Reddy and Guerrero 2004). More recent work has shown that, in addition to plant odors enhancing response to pheromones, pheromone odors can alter responses to plant volatiles (Chaffiol et al. 2014). Thus, plant compounds are sometimes used as pheromones by insects, and pheromones can alter insect responses to plant compounds. However, our discovery that plants can produce bee mandibular pheromone components in nectar, and that these components alter how bees respond to toxic nectar components, opens up new avenues for understanding the complexity of how plant-produced volatile compounds can affect pollinator behavior through co-opting the pollinator's own pheromones.

The widespread overlap in the volatile organic compounds produced by plants and insects (Schiestl 2010) suggests that these bee-flower interactions may occur even without previous co-evolutionary interactions. Our finding of bee mandibular pheromones in the nectars of two distantly related plant species, combined with one other recent study (Sugahara et al. 2013), indicates the exciting possibility that the production of honey bee mandibular pheromones by bee-pollinated plants may be a common phenomenon. For example, the active attractive components of the Oriental orchid, *Cymbidium floribundum*, were identified recently as a mixture of 3-hydroxyoctanoic acid (3-HOAA) and 10-hydroxy-(E)-2-decenoic acid (10-HDA) (Sugahara et al. 2013). In this species, the compounds are produced in floral tissue rather than nectar. Interestingly, the compounds were attractive only as a mixture and not individually. Both these compounds also are mandibular gland components of worker honeybees (Sugahara et al. 2013). Given that orchids, composites, and buckwheat are quite distantly related angiosperms, the presence of insect pheromones in floral scents may be a

widespread phenomenon that occurs beyond specialized co-evolutionary relationships.

Acknowledgments We thank Mr. Yong Xiong and Yonghe Miao for assistance in nectar sampling, and many research assistants including Guangyin Zhao, Jianjun Li, Mingyue Ji, and Liyun Luo. We also thank Dr. Wei Cao of Northwest University, Xi'an City, China, for conducting nectar chemistry analysis, and members of the Adler lab, Prof. David Denlinger, Dr. Martin Heil, and one anonymous reviewer for feedback on the manuscript. This work was supported by National Basic Research Program of China (2007CB411603) and National Natural Science Foundation of China (30870445 and 31270525).

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