

RESEARCH ARTICLE

Appetitive behavior of the honey bee *Apis mellifera* in response to phenolic compounds naturally found in nectars

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ABSTRACT

The honey bee is the most frequently used species in pollination services for diverse crops. In onion crops (*Allium cepa*), however, bees avoid visiting certain varieties, being attracted differently to male sterile (MS) and fertile (OP) lines. These differences might be based on the phenolic profiles of the cultivars' nectars. To understand the relationship between nectar composition and pollinator attraction to different onion lines, we tested sensory and cognitive abilities and palatability in honey bees exposed to MS and OP onion nectars and sugar solutions mimicking them. We evaluated the proboscis extension response (PER) after antennal contact (unconditioned response) to MS or OP onion nectars, finding no statistical differences, which indicates similar gustatory perception for the two nectars. We also performed food uptake assays to test palatability of different artificial nectars, considering their flavonoids and potassium content. The presence of potassium decreased the palatability of the artificial nectars. Finally, we evaluated the bees' cognitive abilities when the reward (unconditioned stimulus) offered during conditioning PER assays presents differences in composition. We found that potassium by itself impaired learning; however, such impairment was even higher when naringenin and quercetin were added in the unconditioned stimulus (MS nectar mimic). Interestingly, potassium together with luteolin (OP nectar mimic) improved learning. Our study demonstrates that the differences in the nectars' flavonoid profiles combined with their high potassium content could explain the previously reported differences in attractiveness between onion lines, suggesting an important role of nectar compounds other than sugars for the attractiveness of flowers to pollinators.

KEY WORDS: Olfactory learning, Responsiveness, Food uptake, Onion nectar, Flavonoid, Pollination

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INTRODUCTION

Flavonoids occur widely in plants and are a biologically major and chemically diverse group of secondary metabolites that possess a wide range of biological activities. They have been widely studied because of their contribution to human health. Nevertheless, flavonoids are also beneficial for the plant itself as physiological active compounds, as stress-protecting agents, as attractants or as feeding deterrents and, in general, by their significant role in plant resistance to disease (Treutter, 2006). Phenolics occur in a large proportion of floral nectars and are often consumed by pollinators (Hagler and Buchmann, 1993). However, these compounds are usually associated with resistance to herbivory (Baker, 1977; Guerrant and Fiedler, 1981) and have been assumed to act primarily as deterrents (Liu et al., 2007). In this sense, their presence in nectar is not completely understood. Many hypotheses have been proposed to explain this issue, including encouraging specialist pollinators, deterring nectar robbers (Feinsinger and Swann, 1978) or preventing microbial degradation (Hagler and Buchmann, 1993).

In the case of onion crops, there is a clear relationship between the phenolic profile of the nectar and honey bee appetitive behavior. Soto and collaborators (2013) found that some phenolic (flavonoid) compounds have a different effect on pollinator preferences, acting as attractants or as repellents. Evidence suggests that insects are able to discriminate among plants with different flavonoid profiles (Simmonds, 2001).

As a worldwide important vegetable crop, onion (*Allium cepa* L.) is an allogamous species that requires insect pollination in order to produce seeds. As a consequence, any significant increase in onion seed production depends markedly on pollination efficiency (Abrol, 2010). Depending on the location of the study, numerous native insect species have been found to visit onion flowers for nectar and pollen (Bohart et al., 1970; Benedek and Gaál, 1972; Nye et al., 1973). However, honey bees (*Apis mellifera* L.) have been reported to be one of the most efficient and major onion pollinators (Nye et al., 1973; Ewies and El Sahhar, 1977) and their hives are commonly borrowed to improve seed yields (Voss et al., 1999).

Two onion varieties are usually grown around the world: fertile or open-pollinated (OP) and first-generation (F1) hybrids. To produce F1 hybrid onion seed, it is necessary to cross a fertile (OP) line with a male-sterile (MS) one. Field observations indicate that F1 hybrid seed yields are much lower than OP variety seed yields, with a decrease of up to 60% (Céspedes et al., 2004). It is assumed that these differences in yield are due to pollination problems owing to the low attractiveness of MS lines (Parker, 1982).

Several previous studies have evaluated factors contributing to the lack of pollinator visits to onion flowers and their subsequent low seed yield. However, the findings of these studies were conflicting or inconclusive. To explain the onion flower's unattractiveness, the high potassium and low sucrose concentrations found in its nectar compared with food rewards

from competing crops have been proposed previously (Lederhouse et al., 1968; Bohart et al., 1970; Nye, 1970; Carlson, 1974; Waller et al., 1974; Gary et al., 1977a,b; Hagler et al., 1990; Silva and Dean, 2000). Waller and co-workers (1974) determined that onion nectar has a high potassium content, about 10 times greater than that of competing flora. Also, Hagler (1990) determined the potassium content in five onion cultivars and found that it ranged between 5347 and 6914 ppm. Both studies found that bee foraging was inversely proportional to the higher potassium concentrations. They thus concluded that these high potassium levels are the primary factor limiting successful bee pollination of onions.

However, these investigations did not explain the documented cultivar differences in onion flower attractiveness. Some studies demonstrated that bees forage selectively among onion species and cultivars (Carlson, 1974; Hagler et al., 1990). Floral nectar composition has been studied as the potential cause of bee preference for certain onion flowers (Waller, 1972; Butler, 1945; Hagler et al., 1990; Waller et al., 1974). Silva and Dean (2000) demonstrated that neither nectar potassium concentration nor onion sugar composition influenced bee behavior.

Soto and co-workers (2013) showed that there are differences in nectar flavonoid profile not only between MS and OP lines but also within MS lines. As stated above, some flavonoids might act as attractants and others as repellents. Specifically, the higher number of bee visits and, as a consequence, the higher seed yield in OP lines may be due to the presence of luteolin (7.2 mg l^{-1}). In contrast, a line that had a high naringenin and quercetin content (MSL1, 8.0 and 1.0 mg l^{-1} , respectively) showed the least number of bee visits. This study also showed that the high potassium content, ranging from 2790 to 3358 ppm, had no relationship with fitness between onion lines, as Silva and Dean (2000) had shown previously.

The honey bee is not only the most abundant pollinator of crops (Williams and Free, 1974) but also a versatile experimental model to evaluate the subtle differences found in different onion nectars under controlled laboratory conditions such as used for other crop rewards (Wright et al., 2013). Bearing this in mind, an experimental approach was proposed to find out differences between the compounds found in onion nectar, in terms of not only their palatability but also their effect on bee sensory and cognitive abilities. Specifically, we aimed to study (i) the unconditioned response of bees to the nectar from MS and OP lines, (ii) the palatability of different phenolic compounds present in the nectar of diverse onion lines and (iii) the bees' cognitive abilities when the reward (unconditioned stimulus) offered during conditioning assays contains different onion nectar compounds.

MATERIALS AND METHODS

Study site, animals and chemical compounds

All experiments were carried out at the Experimental Field of the University of Buenos Aires, Argentina ($34^{\circ}32'S$, $58^{\circ}26'W$) during the summer of 2017. Honey bee foragers were captured at the entrance of 10-frame Langstroth hives formed by a mated queen, three or four frames of capped brood, food reserves and about 20,000 individuals.

The chemical compounds used to prepare the different treatments (see Table 1) were luteolin, quercetin, naringenin and potassium (KCl, P9541; all from Sigma-Aldrich, Steinheim, Germany). To prepare the different solutions, we considered the flavonoid concentrations previously reported by Soto and collaborators (2013).

For conditioning assays, we used hexanol and nonanal (Sigma-Aldrich), both pure odors commonly present in floral fragrances (Knudsen and Tollsten, 1993; Raguso and Pichersky, 1999).

Table 1. Composition and concentration of the solutions used for the different treatments

Treatment	Composition	Concentration	Mimicking
Control	Sucrose solution	50% w/w	
	K ⁺	3000 ppm	
Lut+K ⁺	Sucrose solution	50% w/w	OP onion line
	KCl	3000 ppm	
	Luteolin	10 ppm	
Nar+K ⁺	Sucrose solution	50% w/w	
	KCl	3000 ppm	
	Naringenin	10 ppm	
Quer+K ⁺	Sucrose solution	50% w/w	
	KCl	3000 ppm	
	Quercetin	1 ppm	
Nar+Quer+K ⁺	Sucrose solution	50% w/w	MS onion line
	KCl	3000 ppm	
	Naringenin	10 ppm	
	Quercetin	1 ppm	

Solutions were prepared according to the concentrations naturally found in onion nectar, reported by Soto et al. (2013). KCl was used to obtain the K⁺ concentration.

Nectar extraction

Nectar samples were obtained from an OP line and a MS line cultivated in Luján de Cuyo, Mendoza, Argentina. The onion plants bloomed from November to December 2017. Flowers were picked up at random during anthesis from both lines when at least 50% of the umbels had half of their flowers opened. Nectar extraction was carried out following Soto et al. (2016). Ten freshly opened flowers from umbels were put inside 1.5 ml microtubes after removing anthers, filaments and peduncle. Microtubes were immediately centrifuged (13,000 rpm, 30 min, 4°C). It was possible to extract around 10 µl of nectar from each umbel. These nectar samples were used in experiment 1 for the evaluation of the bees' unconditioned response (see below).

Bee capture and harnessing

Experimental bees of unknown age were randomly captured at the hive entrance. Bees were anesthetized at -4°C for 1 min and confined in wooden cages ($7 \times 7 \times 2.5 \text{ cm}$) in groups of 10 individuals (experiment 2) or individually harnessed in metal tubes (experiments 1 and 3). Cold anesthetization was carried out to reduce stress levels and increase the survival rate (I.G.H. and F.P., personal observation during bee manipulation). Afterwards, bees were kept in darkness in an incubator at 25°C and 75% relative humidity, for 1 h, prior to the experiments.

Harnessing restrained the bees' body movement but allowed them to freely move their antennae and mouthparts (Takeda, 1961).

Behavioral assays

Experiment 1: unconditioned response to onion nectar

Harnessed bees were divided into two groups (OP and MS). At least 40 bees per group were used in this experiment. The reflexive extension of the proboscis after antennal contact (unconditioned response) with the different solutions, given at random, was evaluated. Natural OP line nectar and an artificial sucrose solution with the same sucrose concentration as OP line nectar were used for the OP group; natural MS line nectar and an artificial sucrose solution with the same sucrose concentration as MS line nectar were used for the MS group. Afterwards, the bees' antennae were touched with 50% sucrose solution as a positive control. Only bees that extended their proboscis in response to this control solution were included in the analysis. Three possible responses for each bee were

evaluated: (1) response to the nectar only and not to the sucrose solution; (2) response to the sucrose solution only and not to the nectar; and (3) response to both the nectar and sucrose solution.

Experiment 2: palatability to onion nectar compounds

We randomly captured 60 honey bee foragers at the entrance of five hives and allocated them into groups of 10 individuals for each of the different treatments. As a first step, in order to equilibrate the initial motivation to consume food, bees were fed *ad libitum* with 1.8 mol l⁻¹ sucrose solution (50% w/w) for 24 h. The sugar solution consumption and the survival rates were registered. After this, the diet was changed, and bees were offered one of the treatments described in Table 1. After 24 h, the percentage survival and the ingested volume (consumption) of the different offered solutions per bee were quantified. A total of 10 cages per treatment were evaluated.

Experiment 3: olfactory classical conditioning of proboscis extension

To study whether the compounds present in the different onion nectars play a differential role in terms of reward, bees were harnessed and underwent a classical conditioning protocol adapted from the proboscis extension response (PER) paradigm (Takeda, 1961; Bitterman et al., 1983). During a conditioning procedure, bees learn to associate a given olfactory stimulus (conditioned stimulus, CS) with a reward (unconditioned stimulus, US). To assay the PER, a device that delivered a continuous airflow (50 ml s⁻¹) was used for the application of the odorant; 4 µl of pure odorant impregnated on 30×3 mm filter paper inside a syringe were delivered through a secondary airstream (6.25 ml s⁻¹) to the head of the bee. A fan extracted the released odors to avoid contamination. Bees underwent 5 training trials of paired CS–US presentations, with an inter-trial interval between CS presentations of 15 min. Each learning trial lasted 39 s. Before odor presentation, bees rested for 16 s in the airflow for familiarization as well as for testing the bees' response towards the mechanical stimulus. Only bees that did not respond to the mechanical airflow stimulus were used. For the classical conditioning training procedure, the CS was presented for 6 s. Reinforcement (according to the treatment) was presented for 3 s on the proboscis (mouthparts), 3 s after the onset of the CS. After odor presentation, the learning trial ended with 17 s of clean airflow. To evaluate whether the bees had formed a medium-term memory after the learning assay, bees stayed harnessed for 15 min and were then subjected to: (1) the presentation of the CS and (2) the presentation of the novel odor, both without reinforcement. The presentation order of the odors during the tests was balanced and a time gap of 15 min was used between each presentation. The PER was considered during the first 3 s of the presentation of the test odor.

In a first experimental series, nonanal was used as the CS and a second pure odor, hexanol, was used as a novel odor during the testing phase. As US, one of the treatments was used (see Table 1). As a second experimental series, we performed an olfactory classical conditioning of proboscis extension in honey bees in order to analyze the effect of the most common onion nectar compounds without the presence of potassium. In this case, we evaluated the bees' response towards one of three treatments: (1) 1.8 mol l⁻¹ sucrose solution (control, 50% w/w), (2) 1.8 mol l⁻¹ sucrose solution and 10 mg luteolin per liter of sucrose solution (Lut) or (3) 1.8 mol l⁻¹ sucrose solution and 10 mg naringenin per liter of sucrose solution (Nar).

Statistical analysis

All statistical tests were performed with R v3.3.3 (<http://www.R-project.org/>). The unconditioned response towards the different

nectars was analyzed throughout an index obtained from the bees' response to each nectar onion line minus the response to its respective sucrose solution (with the same sugar concentration). Differences between treatments (nectar onion lines: OP or MS) were analyzed with Fisher's exact test (McDonald, 2014).

The volume ingested by 10 caged bees fed with different nectar compounds was analyzed by means of generalized linear models (GLM) following a gamma error distribution and using the *glm* function of the *lme4* package (Bates et al., 2015). Treatment (a six-level factor) was considered as a fixed factor.

The PER in the conditioning assays was assessed by means of generalized linear mixed-effect models (GLMM) and generalized linear models (GLM), following a binomial error distribution and using the *glmer* and *glm* functions of the *lme4* package (Bates et al., 2015). In the case of training, treatment (a six-level factor corresponding to the different treatments for the first experimental series: control, K⁺, Lut+K⁺, Nar+K⁺, Quer+K⁺, Nar+Quer+K⁺; a three-level factor corresponding to the different treatments for the second experimental series: control, Lut and Nar) and trials (a four-level factor corresponding to 2–5 trials) were considered as fixed effects, with each bee included as a random factor. In the case of test phase, we analyzed the response to the CS, considering only those bees that did not extend their proboscis towards the novel odor. This was done in order to distinguish responses that were odor specific to nonanal (CS). We took into account the treatment (a six-level factor corresponding to the different treatments for the first experimental series: control, K⁺, Lut+K⁺, Nar+K⁺, Quer+K⁺, Nar+Quer+K⁺; a three-level factor corresponding to the different treatments for the second experimental series: control, Lut and Nar) as a fixed effect. GLM and GLMM were simplified as follows: significance of the different terms was tested starting from the higher-order terms model using *anova* function to compare between models (Chambers and Hastie, 1992). Non-significant terms (*P*>0.05) were removed (see Tables S1–S4).

RESULTS

Experiment 1: unconditioned response to onion nectars

When we analyzed the bees' unconditioned extension of the proboscis in response to nectars extracted from OP and MS onion lines, we found no significant differences in the percentage of bees responding (Fig. 1). The estimated sugar concentrations of the different onion lines, OP and MS, were 26% w/w and 33% w/w, respectively. The proportion of bees that responded only to the onion nectar or only to the sucrose solution with the same sugar concentration as the nectars offered, or to both (onion nectar and sucrose solution separately) was similar between the OP and the MS groups (Fisher's exact test, *P*=0.8127).

Experiment 2: palatability of onion nectar compounds

The palatability of different sucrose solutions to caged bees was analyzed by finding significant differences in the ingested volume between the control and the different treatments (Fig. 2; minimal adequate model: Ingested volume~Treatment; *P*=0.01306; Table S1). Bees fed with pure sugar solution (50% w/w) consumed 31±3 µl per bee, while bees fed with the different treatments consumed 20±1 µl per bee (Fig. 2). However, no significant differences were found in the ingested volume between the different treatments.

The consumption of pure sugar solution (50% w/w) by bees in each of the treatment groups was analyzed prior to offering the different diets. No statistical differences between the different treatment groups were found (56±2 µl per bee; minimal adequate model: Ingested volume~1; Table S1).

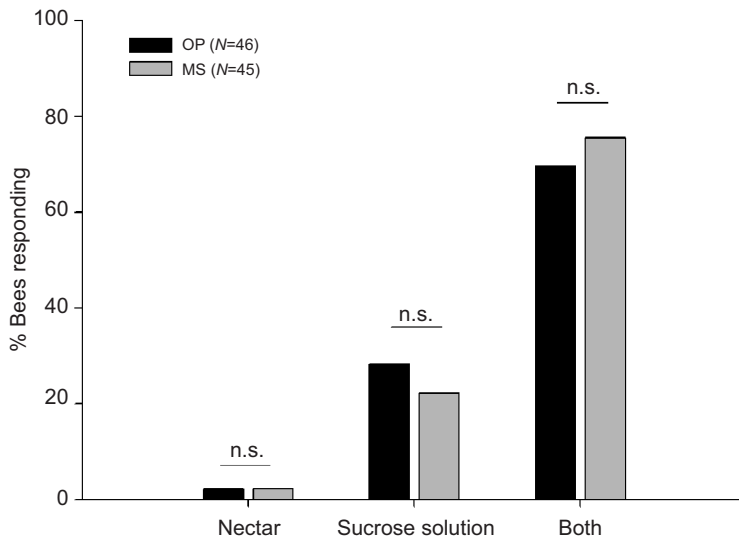


Fig. 1. Unconditioned response of honey bees to onion nectars. Percentage of worker bees that responded by extending their proboscis to the presentation of the onion nectar only (OP or MS), to sucrose solutions of the same concentration as these nectars only (26% w/w sucrose solution for OP nectar and 33% w/w for MS nectar) or to both (nectar and sugar solution separately) onto their antennae. No significant differences were found between the onion nectars ($P=0.8127$, n.s.).

Experiment 3: olfactory classical conditioning of proboscis extension

The analysis of the effect of the different compounds as a reward (US) showed significant differences between treatments in learning performance during olfactory classical conditioning (Fig. 3). In the acquisition phase, the proportion of bees responding to the CS (nonanal) increased with successive conditioning trials and differed between treatments (Fig. 3, left panel; minimal adequate model: $\text{Response} \sim \text{Treatment} + \text{Trial} + 1 | \text{ind.}$, $P < 0.001$; Table S1). In the 4th trial, bees rewarded with sugar solution (50% w/w) alone (control), K^+ or $\text{Lut} + \text{K}^+$ had response levels that were not significantly different (76.5%, 67.6% and 83.8%, respectively), but that differed from those of bees rewarded with $\text{Nar} + \text{K}^+$, $\text{Quer} + \text{K}^+$ or $\text{Nar} + \text{Quer} + \text{K}^+$, which exhibited significantly lower levels of acquisition (42.2%, 40.5% and 27.5%, respectively; Table S2). In the 5th trial, the performance of bees rewarded with K^+ decreased drastically

(52.9%) while that of bees rewarded with $\text{Quer} + \text{K}^+$ showed an increase (56.7%; Table S3).

As the response to a novel odor is a control to evaluate whether the bees established the correct association during the training phase, the bee's response between treatments against the CS (nonanal) was analyzed, taking into account only those bees that did not extend their proboscis towards the novel odor (hexanol) (Fig. 3, right panel). Bees showed a significantly different response between treatments (minimal adequate model: $\text{Response} \sim \text{Treatment}$, $P = 2.287 \times 10^{-7}$; Tables S1 and S4).

When we analyzed the effect of onion nectar compounds on olfactory classical conditioning of proboscis extension in honey bees, without the presence of potassium, we found no significant differences between treatments (Fig. 4; in the training phase: Minimal adequate model: $\text{Response} \sim \text{Trial} + 1 | \text{ind.}$, $P = 0.02214$, Table S1; in the testing phase: Minimal adequate model: $\text{Response} \sim 1$, $P = 0.4334$).

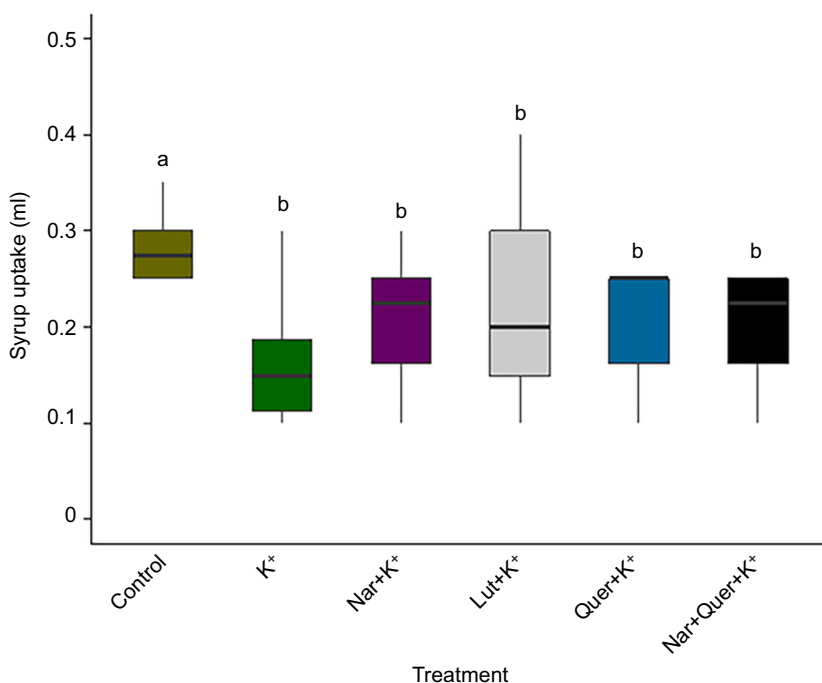


Fig. 2. Effect of onion nectar compounds on syrup uptake by caged honey bees. Total volume of sucrose solution ingested per 10 bees after offering 1.8 mol l^{-1} sucrose solution alone (control, 50% w/w) or with: potassium (K^+); potassium and naringenin ($\text{Nar} + \text{K}^+$); potassium and luteolin ($\text{Lut} + \text{K}^+$); potassium and quercetin ($\text{Quer} + \text{K}^+$); or potassium, naringenin and quercetin ($\text{Nar} + \text{Quer} + \text{K}^+$; see Materials and Methods for composition). Bees fed with sucrose solution alone ingested a greater volume than bees fed with the other treatments (minimal adequate model: $\text{Ingested volume} \sim \text{Treatment}$; $P = 0.01306$; Table S1). Different letters indicate significant differences between treatments.

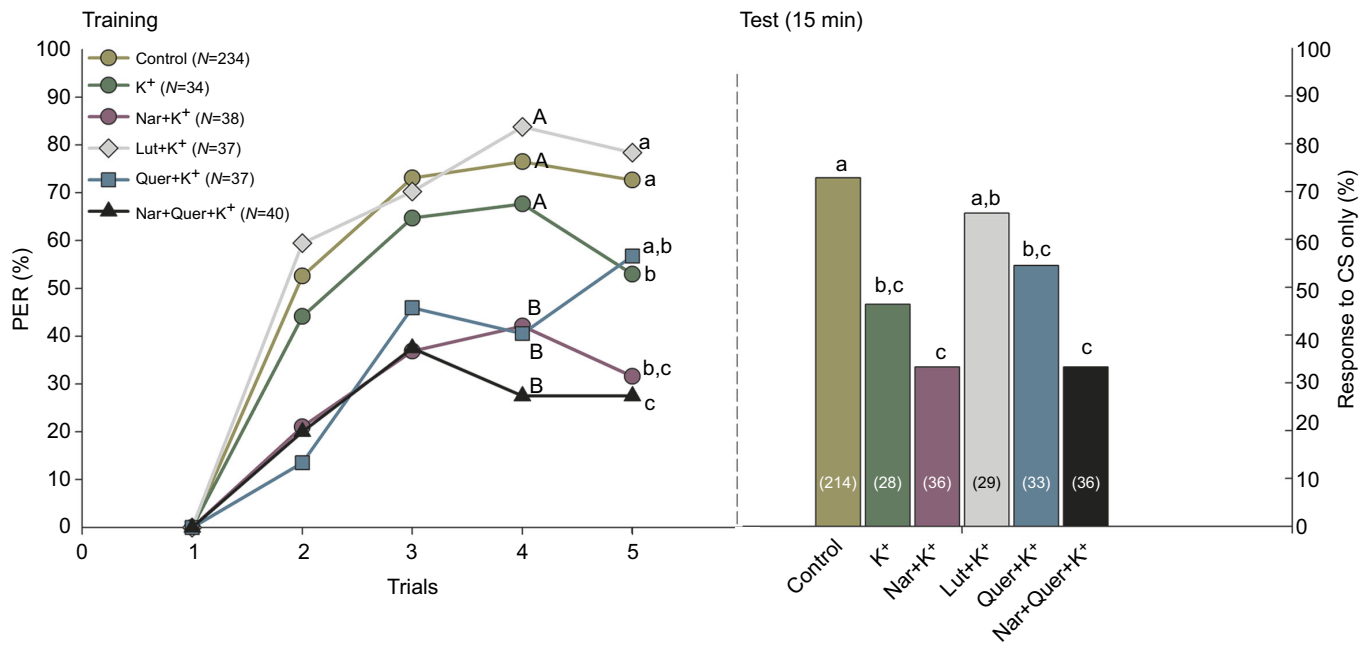


Fig. 3. Effect of onion nectar compounds on olfactory classical conditioning of proboscis extension in honey bees. The proboscis extension response (PER) towards the trained odor was quantified over the course of 5 acquisition trials (Training, left) in which the unconditioned stimulus (US) consisted of 1.8 mol l^{-1} sucrose solution alone (control, 50% w/w) or with: K⁺; Nar+K⁺; Lut+K⁺; Quer+K⁺; or Nar+Quer+K⁺ (see Materials and Methods for composition). In the training phase, the proportion of bees responding to the CS (nonanal) increased with successive conditioning trials and differed between treatments (minimal adequate model: $\text{Response} \sim \text{Treatment} + \text{Trial} + 1 | \text{ind.}$, $P < 0.001$; Table S1). Different capital or lowercase letters indicate significant differences between treatments in the 4th or 5th trial, respectively. In the testing phase (Test, right), the proportion of bees that responded only to the CS (nonanal) was significantly different between treatments (minimal adequate model: $\text{Response} \sim \text{Treatment}$, $P = 2.287 \times 10^{-7}$; Table S1). Different letters indicate significant differences (see Table S4). The number of bees tested is shown in parentheses.

DISCUSSION

Our study shows that the previously reported differential preference of honey bees for different onion lines can be explained by the interaction among chemical compounds found in the nectars of the

different onion lines. The unconditioned responses to both OP and MS onion nectars showed no difference in terms of gustatory responsiveness. This suggests that the short-term response to the chemosensory stimuli of the tested nectars is not affected by the

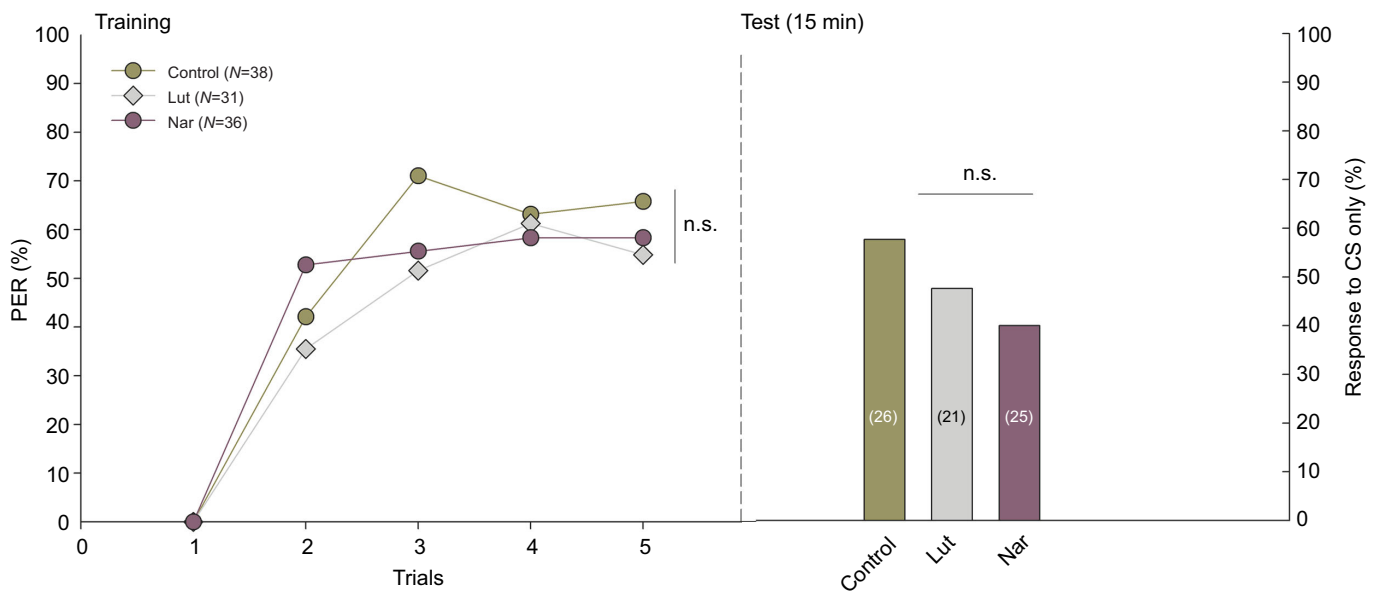


Fig. 4. Effect of onion nectar compounds on olfactory classical conditioning of proboscis extension in honey bees in the absence of potassium. The PER towards the trained odor was quantified over the course of 5 acquisition trials (Training, left) in which the US consisted of 1.8 mol l^{-1} sucrose solution alone (control, 50% w/w) or with Lut or Nar (see Materials and Methods for composition). In the training phase, the proportion of bees responding to the CS (nonanal) increased only with successive conditioning trials, without significant differences between treatments (minimal adequate model: $\text{Response} \sim \text{Trial} + 1 | \text{ind.}$, $P = 0.02214$; Table S1). In the testing phase (Test, right), no significant differences were found between treatments (minimal adequate model: $\text{Response} \sim 1$, $P = 0.5963$). n.s., no significant difference. The number of bees tested is shown in parentheses.

onion lines. However, bees reared with a diet containing potassium showed a reduced food uptake, pointing to impaired palatability. More subtle effects, yet with relevant consequences for resource preference, were also found during olfactory learning processes in PER assays. Learning performance changed dramatically depending on the flavonoid traces added to a sugar solution containing potassium. If the reinforcement offered during the learning procedure contained luteolin (OP-mimic onion nectar), the learning performance and memory retention were enhanced, but when the flavonoids added were naringenin, quercetin or both (MS-mimic nectar), learning performance decreased. All these responses as a whole are consistent with the biased preferences reported for onion lines containing luteolin as compared with other flavonoids.

Gustatory perception and palatability

As a first approach to understanding previously informed differences in bee visits to MS and OP lines, we evaluated the unconditioned response to both nectars. Our experimental results show that there were no statistical differences in the unconditioned response to OP and MS nectars. Based on this, previously reported differences in bee visits between OP and MS lines cannot be explained.

After analyzing the palatability of different phenolic compounds together with potassium, we found that the syrup uptake was significantly higher in the control (sugar solution 50% w/w) compared with the other treatments. We did not find any significant difference between the treatments, excluding the control, denoting a clear effect of the potassium presence. However, these results on palatability do not explain the difference previously observed in the attractiveness of flowers from diverse onion lines.

The analysis of the palatability of the different nectar compounds strengthens the view that potassium concentration plays a relevant role in reducing nectar palatability. This has been previously reported by Hagler (1990); however, Silva and Dean (2000) and Soto and collaborators (2013) reported that potassium concentration is not an important factor comparing visits between different onion lines. This could be explained by the fact that onion nectar has extremely high potassium concentrations in both MS and OP (fertile) lines. Taking into account this information, it could be said that potassium concentration alone cannot explain differences between different lines; nevertheless, it could explain why it has been reported that bees usually prefer foraging at competing flora (Gary et al., 1977a). In this sense, the palatability results show a good match to the low preference for the onion flowers previously reported.

Learning ability and onion nectar contents

In animal-pollinated plants, fitness is influenced by floral traits that function as an advertisement and as a reward for pollinators (Poveda et al., 2005). Flowers present different structures that must be learned by pollinators by trial and error for acquiring food. All this information is used by them to choose between flowers of different species and to make foraging decisions (Abrol, 2006). In this way, minor components of nectar might directly affect bees' foraging activity. Concerning the bees' cognitive abilities, we found interesting results showing that the high potassium concentration tested in the present study triggered a decrement in bee learning performance. Moreover, this negative effect was increased with the addition of naringenin and quercetin. It has been reported that the presence of naringenin is correlated with low bee visits (Soto et al., 2013). In this sense, it is interesting to mention that naringenin and quercetin were detected in MS lines only. It is well documented that MS lines are less visited by bees than fertile ones. Although these differences in attractiveness between MS and OP lines have been

previously attributed to some floral traits of MS lines such as the absence of pollen, lower nectar volume, a longer style and smaller tepals (Silva and Dean, 2000; Soto et al., 2013), these results also suggest that the presence of traces of certain compounds like naringenin and quercetin in MS nectar combined with the effect of potassium could also contribute to this lower visits rate. However, our results are not consistent with those reported by Liao and collaborators (2017), who demonstrated that bees displayed a preference response to quercetin (0.01, 0.05, 0.10, 0.25 and 0.50 mmol l⁻¹) according to visitation frequency and consumption ratios, in semi-field free-flight experiments. Nevertheless, it should be noted that contrary to the work previously mentioned, in our study, a decrement in learning performance was observed in the presence of potassium and with a much lower quercetin concentration (0.003 mmol l⁻¹=1 ppm), corresponding to the level found in the nectar of the MS line. This shows that the effects of the flavonoid quercetin can be modified by the presence of potassium. The same is true of luteolin, which, as we have shown, enhances the bees' learning, but only in the presence of potassium. These differences in the observed effect due to the combination of compounds have already been reported in previous studies where other flavonoids were considered (Liu et al., 2007; Onyilagha et al., 2012).

Flavonoids as key compounds for insect food preference

Regarding the effect caused by the different flavonoids, different authors have reported contrasting effects in insects, probably because plants use these compounds as a defense against herbivores. On the one hand, the compounds can present insecticidal activity, affecting the feeding behavior and development of moths (Morimoto et al., 2000, 2003; Anshul et al., 2013), the feeding behavior of beetles (Onyilagha et al., 2012) and the feeding behavior and development of aphids (Ateyyat et al., 2012; Goławska and Łukasik, 2012; Goławska et al., 2014). Additionally, Goławska and Łukasik (2012) demonstrated that the flavonoid luteolin affected pea aphid feeding behavior, reducing aphid ingestion. Moreover, Goławska and collaborators (2014) reported that increasing the concentration of the flavonoids naringenin and quercetin in the liquid artificial diet significantly decreased fecundity and increased mortality of adult apterae aphids. On the other hand, it has been demonstrated that quercetin has some health benefits for bees, including the up-regulation of detoxification and immunity genes (Mao et al., 2013, 2017).

Most plants contain a large array of flavonoids, and evidence suggests that insects are able to discriminate among plants with different flavonoid profiles (Simmonds, 2001). Secondary compounds commonly associated with herbivore defense have been found in the nectar of many plant species, and many plants produce nectar that is toxic or repellent to some floral visitors (Adler, 2001). However, the presence of certain flavonoid compounds in the nectar of some species that may be repellent to pollinators is not totally understood. These compounds may be transported into nectar due to their presence in phloem rather than as a consequence of a positive selection by pollinators (Adler, 2001). In the case of crops such as onion, the presence of some deterrent compounds in nectar may be due to the artificial selection of other positive characteristics.

When bees and other pollinators learn to associate floral scent with food while foraging, they are more likely to visit flowers bearing the same scent signals (Wright and Schiestl, 2009). Such behavior increases their foraging efficiency (Chittka et al., 1997) while concomitantly leading to more effective pollination (Kunin, 1993; Hopkins and Rausher, 2012). In this context, it is worth

mentioning that artificial selection taking into account the flavonoid profiles of onion nectars could help to increase yields of hybrid seed production because, as we have shown, flavonoids can affect the responses of pollinators to flowers. In conclusion, it is fundamental to point out the relevance of nectar compounds other than sugars for the floral attractiveness to pollinators.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: I.G., F.P., W.M.F.; Methodology: I.G., F.P.; Validation: I.G., F.P., I.M.; Formal analysis: I.G., F.P., I.M.; Investigation: I.G., F.P., I.M.; Resources: W.M.F.; Writing - original draft: I.G., F.P., W.M.F.; Writing - review & editing: I.G., F.P., C.R.G., W.M.F.; Visualization: W.M.F.; Supervision: W.M.F.; Project administration: C.R.G., W.M.F.; Funding acquisition: C.R.G., W.M.F.

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Supplementary information

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References

- Abrol, D. P. (2006). Foraging behaviour of bees as influenced by quality and quantity of rewards from flowers. *J. Asia Pacific Entomol.* **9**, 1-4.
- Abrol, D. P. (2010). Foraging behaviour of *Apis florea* F., an important pollinator of *Allium cepa* L. *J. Apic. Res.* **49**, 318-325.
- Adler, L. S. (2001). The ecological significance of toxic nectar. *Oikos* **91**, 409-420.
- Anshul, N., Bhakuni, R. S., Gaur, R. and Singh, D. (2013). Isomeric Flavonoids of *Artemisia annua* (Asterales: Asteraceae) as Insect Growth Inhibitors Against *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Fla. Entomol.* **96**, 897-903.
- Ateyyat, M., Abu-Romman, S., Abu-Darwish, M. and Ghabeish, I. (2012). Impact of flavonoids against woolly apple aphid, *Eriosoma lanigerum* (Hausmann) and its sole parasitoid, *Aphelinus mali* (Hald). *J. Agric. Sci.* **4**, 227-236.
- Baker, H. G. (1977). Non-sugar chemical constituents of nectar. *Apidologie* **8**, 349-356.
- Bates, D., Maechler, M., Bolker, B. and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 1-48.
- Benedek, P. and Gaál, E. (1972). The effect of insect pollination on seed onion, with observations on the behaviour of honeybees on the crop. *J. Apic. Res.* **11**, 175-180.
- Bitterman, M. E., Menzel, R., Fietz, A. and Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol.* **97**, 107-119.
- Bohart, G. E., Nye, W. P. and Hawthorn, L. R. (1970). Onion pollination as affected by different levels of pollinator activity. *Utah Agric. Exp. Stn. Bul.* **482**, 1-57.
- Butler, C. G. (1945). The influence of various physical and biological factors of the environment on honey bee activity: an examination of the relationship between activity and nectar concentration and abundance. *J. Exp. Biol.* **21**, 5-12.
- Carlson, E. (1974). Onion varieties, honey bee visitations, and seed yield. *Calif. Agric.* **28**, 16-18.
- Céspedes, M., Peralta, I. and Galmarini, C. (2004). Relación entre la morfología de la flor de cebolla y la frecuencia de visitas de abejas polinizadoras. *Hortic. Argent.* **23**, 1-65.
- Chambers, J. M. and Hastie, T. J. (1992). *Statistical Models in S*. New York, NY: Chapman and Hall.
- Chittka, L., Gumbert, A. and Kunze, J. (1997). Foraging dynamics of bumble bees: Correlates of movements within and between plant species. *Behav. Ecol.* **8**, 239-249.
- Ewies, M. A. and El-Sahhar, K. F. (1977). Observations on the behaviour of honeybees on onion and their effects on seed yield. *J. Apic. Res.* **16**, 194-196.
- Feinsinger, P. and Swarm, L. A. (1978). How common are ant-repellent nectars. *Biotropica* **10**, 238-239.
- Gary, N. E., Witherell, P. C., Lorenzen, K. and Marston, J. M. (1977a). The interfield distribution of honey bees foraging on carrots, onions, and safflower. *Environ. Entomol.* **6**, 637-640.
- Gary, N. E., Witherell, P. C., Lorenzen, K. and Marston, J. M. (1977b). Area fidelity and intra-field distribution of honey bees during the pollination of onions. *Environ. Entomol.* **6**, 303-310.
- Goławska, S. and Łukasik, I. (2012). Antifeedant activity of luteolin and genistein against the pea aphid, *Acyrtosiphon pisum*. *J. Pest Sci.* **85**, 443-450.
- Goławska, S., Sprawka, I., Łukasik, I. and Goławski, A. (2014). Are naringenin and quercetin useful chemicals in pest-management strategies? *J. Pest Sci.* **87**, 173-180.
- Guerrant, E. O., Jr. and Fiedler, P. L. (1981). Flower defenses against nectar-pilferage by ants. *Biotropica* **25**-33.
- Hagler, J. R. (1990). Honeybee (*Apis mellifera*) response to simulated onion nectars containing variable sugar and potassium concentrations. *Apidologie* **21**, 115-121.
- Hagler, J. R. and Buchmann, S. L. (1993). Honey bee (Hymenoptera: Apidae) foraging responses to phenolic-rich nectars. *J. Kansas Entomol. Soc.* **223**-230.
- Hagler, J. R., Cohen, A. and Loper, G. M. (1990). Production and composition of onion nectar and honey bee (Hymenoptera: Apidae) foraging activity in Arizona. *Environ. Entomol.* **19**, 327-331.
- Hopkins, R. and Rausher, M. D. (2012). Pollinator-mediated selection on flower color allele drives reinforcement. *Science* **335**, 1090-1092.
- Knudsen, J. T. and Tollsten, L. (1993). Trends in floral scent chemistry in pollination syndromes: floral scent composition in moth-pollinated taxa. *Bot. J. Linn. Soc.* **113**, 263-284.
- Kunin, W. E. (1993). Sex and the single mustard-population-density and pollinator behavior effects on seed-set. *Ecology* **74**, 2145-2160.
- Lederhouse, R. C., Caron, D. M. and Morse, R. A. (1968). Onion pollination in New York. *N.Y. Food Life Sci.* **1**, 8-9.
- Liao, L.-H., Wu, W.-Y. and Berenbaum, M. R. (2017). Behavioral responses of honey bees (*Apis mellifera*) to natural and synthetic xenobiotics in food. *Sci. Rep.* **7**, 15924.
- Liu, F., Chen, J., Chai, J., Zhang, X., Bai, X., He, D. and Roubik, D. W. (2007). Adaptive functions of defensive plant phenolics and a non-linear bee response to nectar components. *Funct. Ecol.* **21**, 96-100.
- Mao, W., Schuler, M. A. and Berenbaum, M. R. (2013). Honey constituents up-regulate detoxification and immunity genes in the western honey bee *Apis mellifera*. *Proc. Natl. Acad. Sci. USA* **110**, 8842-8846.
- Mao, W., Schuler, M. A. and Berenbaum, M. R. (2017). Disruption of quercetin metabolism by fungicide affects energy production in honey bees (*Apis mellifera*). *Proc. Natl. Acad. Sci. USA* **114**, 2538-2543.
- McDonald, J. H. (2014). *Handbook of Biological Statistics*, 3rd edn. Baltimore, Maryland: Sparky House Publishing.
- Morimoto, M., Kumeda, S. and Komai, K. (2000). Insect antifeedant flavonoids from *Gnaphalium affine* D. Don. *J. Agric. Food Chem.* **48**, 1888-1891.
- Morimoto, M., Tanimoto, K., Nakano, S., Ozaki, T., Nakano, A. and Komai, K. (2003). Insect antifeedant activity of flavones and chromones against *Spodoptera litura*. *J. Agric. Food Chem.* **51**, 389-393.
- Nye, W. P. (1970). Pollination of onion seed affected by environmental stresses. The indispensable pollinators. A report of the ninth pollination conference, hot springs, Ark. *Univ. Ark. Agric. Ext. Serv. MP* **127**, 141-144.
- Nye, W. P., Shasha'a, N. S., Campbell, W. F. and Hamson, A. R. (1973). Insect pollination and seed set of onions (*Allium cepa* L.). *Utah Agric. Exp. Stn. Res. Rep.* **6**, 15.
- Onyilagha, J. C., Gruber, M. Y., Hallett, R. H., Holowachuk, J., Buckner, A. and Soroka, J. J. (2012). Constitutive flavonoids deter flea beetle insect feeding in *Camelina sativa* L. *Biochem. Syst. Ecol.* **42**, 128-133.
- Parker, F. D. (1982). Efficiency of bees in pollinating onion flowers. *J. Kansas Entomol. Soc.* **171**-176.
- Poveda, K., Steffan-Dewenter, I., Scheu, S. and Tschardtke, T. (2005). Floral trait expression and plant fitness in response to below- and aboveground plant-animal interactions. *Perspect. Plant Ecol.* **7**, 77-83.
- Raguso, R. A. and Pichersky, E. (1999). New perspectives in pollination biology: floral fragrances. A day in the life of a linalool molecule: chemical communication in a plant-pollinator system. Part 1: linalool biosynthesis in flowering plants. *Plant Species Biol.* **14**, 95-120.
- Silva, E. M. and Dean, B. B. (2000). Effect of nectar composition and nectar concentration on honey bee (Hymenoptera: Apidae) visitations to hybrid onion flowers. *J. Econ. Entomol.* **93**, 1216-1221.
- Simmonds, M. S. J. (2001). Importance of flavonoids in insect-plant interactions: feeding and oviposition. *Phytochemistry* **56**, 245-252.
- Soto, V. C., Maldonado, I. B., Gil, R. A., Peralta, I. E., Silva, M. F. and Galmarini, C. R. (2013). Nectar and flower traits of different onion male sterile lines related to pollination efficiency and seed yield of F1 hybrids. *J. Econ. Entomol.* **106**, 1386-1394.
- Soto, V. C., Joffré, V. P., Galmarini, C. R. and Silva, M. F. (2016). Determination of alkaloids in onion nectar by micellar electrokinetic chromatography. *Electrophoresis* **37**, 1909-1915.
- Takeda, K. (1961). Classical conditioning response in the honey bee. *J. Insect Physiol.* **6**, 168-179.

- Treutter, D.** (2006). Significance of flavonoids in plant resistance: a review. *Environ. Chem. Lett.* **4**, 147-157.
- Voss, R., Murray, M., Bradford, K., Mayberry, K. and Miller, I.** (1999). *Onion Seed Production in California*. UCANR Publications.
- Waller, G. D.** (1972). Evaluating responses of honey bees to sugar solutions using an artificial flower feeder. *Ann. Entomol. Soc. Am.* **65**, 857-862.
- Waller, G. D., Carpenter, E. W. and Ziehl, O. A.** (1974). Potassium in onion nectar and its probable effects on attractiveness of onion flowers to honey bees. *J. Am. Soc. Hortic. Sci.* **97**, 535-539.
- Williams, I. H. and Free, J. B.** (1974). The pollination of onion (*Allium cepa* L.) to produce hybrid seed. *J. Appl. Ecol.* 409-417.
- Wright, G. A. and Schiestl, F. P.** (2009). The evolution of floral scent: the influence of olfactory learning by insect pollinators on the honest signalling of floral rewards. *Funct. Ecol.* **23**, 841-851.
- Wright, G. A., Baker, D. D., Palmer, M. J., Stabler, D., Mustard, J. A., Power, E. F., Borland, A. M. and Stevenson, P. C.** (2013). Caffeine in floral nectar enhances a pollinator's memory of reward. *Science* **339**, 1202-1204.

Table S1. Set of variables considered in the GL and GLM effects models explaining bees' responses in the different experiments.

Experiment	Phase	Variable	Chi sq	P-values
<i>Palatability of different nectar solutions on caged bees</i>	Control (prior to treatments)	Treatment	1.3563	0.1181
	Treatments	Treatment	3.0976	0.01306
<i>Olfactory classical conditioning of proboscis extension</i>	Training	TrialxTreatment	14.146	0.5145
		Trial	74.527	4.577e⁻¹⁶
		Treatment	145.88	2.2e-16
	Trial 4	Treatment	61.79	5.183e⁻¹²
	Trial 5	Treatment	51.925	5.59e⁻¹⁰
	Test	Treatment	39.081	2.287e⁻⁰⁷
<i>Olfactory classical conditioning of proboscis extension excluding the K⁺ effect</i>	Training	TrialxTreatment	4.1398	0.6578
		Trial	9.6151	0.02214
		Treatment	2.8085	0.2455
	Test	Treatment	1.6723	0.4334

Table S2. Trail 4 of the training phase of the Olfactory classical conditioning of proboscis extension. Z values (below diagonal) and p-values (above diagonal) obtained from comparisons between treatments.

Treatment	SS	K ⁺	Luteolin + K ⁺	Naringenin + K ⁺	Quercetin + K ⁺	Naringenin + Quercetin + K ⁺
SS		0.265895	0.327	3.65e⁻⁰⁵	2.23e⁻⁰⁵	2.61e⁻⁰⁸
K ⁺	1.113		0.1171	0.0319	0.0240	0.000811
Luteolin + K ⁺	0.979	1.567		0.000401	0.000282	4.52e⁻⁰⁶
Naringenin + K ⁺	4.129	2.145	3.539		0.8905	0.1778
Quercetin + K ⁺	4.240	2.257	3.631	0.138		0.2288
Naringenin + Quercetin + K ⁺	5.565	3.349	4.586	1.348	1.203	

Table S3. Trail 5 of the training phase of the Olfactory classical conditioning of proboscis extension. Z values (below diagonal) and p-values (above diagonal) obtained from comparisons between treatments.

Treatment	SS	K ⁺	Luteolin + K ⁺	Naringenin + K ⁺	Quercetin + K ⁺	Naringenin + Quercetin + K ⁺
SS		0.0215	0.4648	3.78e⁻⁰⁶	0.0520	3.81e⁻⁰⁷
K ⁺	2.300		0.0264	0.0689	0.7469	0.0276
Luteolin + K ⁺	0.731	2.221		0.000102	0.0503	2.35e⁻⁰⁵
Naringenin + K ⁺	4.623	1.819	3.886		0.029990	0.6931
Quercetin + K ⁺	1.943	0.323	1.957	2.170		0.01053
Naringenin + Quercetin + K ⁺	5.078	2.203	4.229	0.395	2.558	

Table S4. Test phase of the Olfactory classical conditioning of proboscis extension.
Z values (below diagonal) and p-values (above diagonal) obtained from comparisons between treatments.

Treatment	SS	K ⁺	Luteolin + K ⁺	Naringenin + K ⁺	Quercetin + K ⁺	Naringenin + Quercetin + K ⁺
SS		0.00562	0.40779	1.28 e⁻⁰⁵	0.03459	1.28 e⁻⁰⁵
K ⁺	1.1325		0.14924	0.28854	0.52792	0.28854
Luteolin + K ⁺	0.3476	0.7850		0.0113	0.3807	0.0113
Naringenin + K ⁺	1.6826	0.5500	1.3350		0.0783	1.000
Quercetin + K ⁺	0.8071	0.3254	0.4595	0.8755		0.0783
Naringenin + Quercetin + K ⁺	1.6826	0.5500	1.3350	1.490 e ⁻¹⁵	0.8755	