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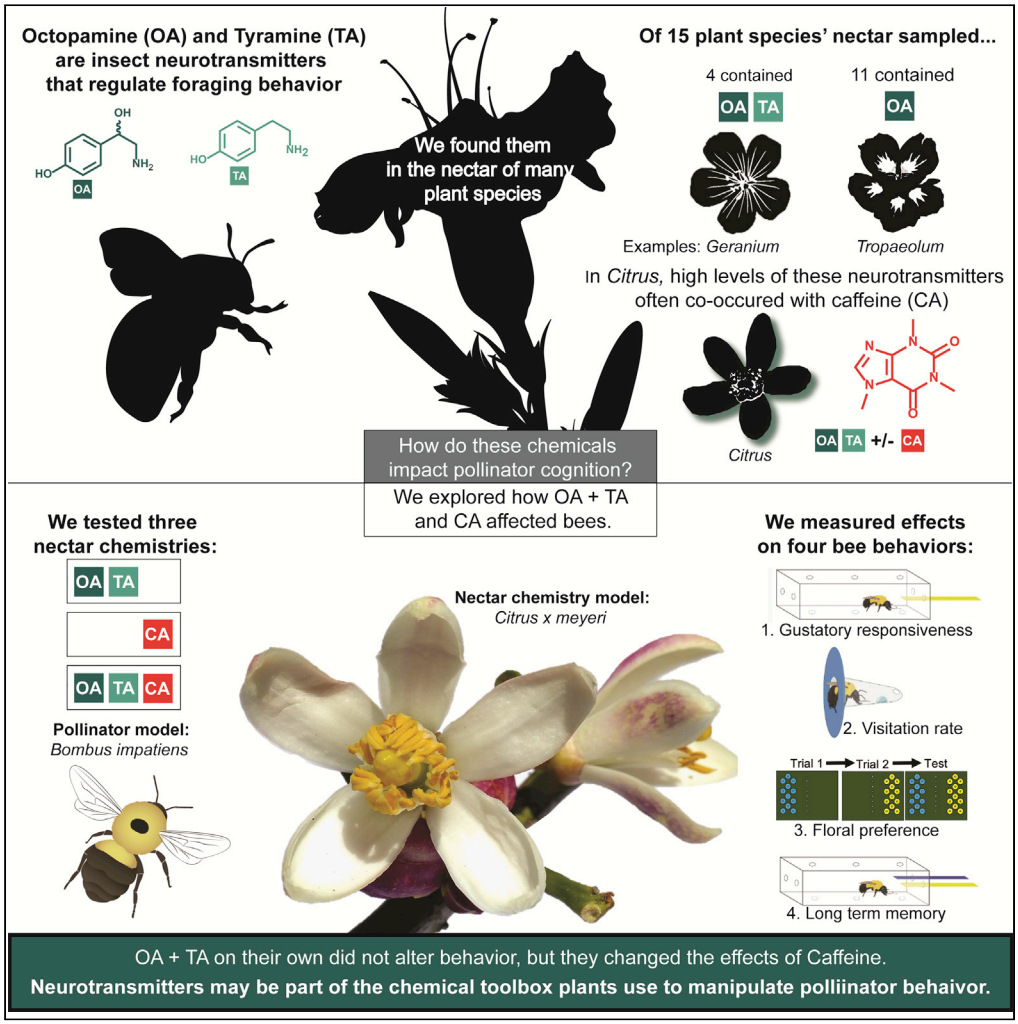

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Highlights

We found octopamine and tyramine in the floral nectar of 15 plant species

These neurotransmitters orchestrate insect foraging and influence bee cognition

In *Citrus*, these chemicals occur with caffeine, well known for its effects on bees

Nectar neurotransmitters interact with caffeine to alter pollinator behavior

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Discovery of octopamine and tyramine in nectar and their effects on bumblebee behavior

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SUMMARY

Nectar chemistry can influence the behavior of pollinators in ways that affect pollen transfer, yet basic questions about how nectar chemical diversity impacts plant-pollinator relationships remain unexplored. For example, plants' capacity to produce neurotransmitters and endocrine disruptors may offer a means to manipulate pollinator behavior. We surveyed 15 plant species and discovered that two insect neurotransmitters, octopamine and tyramine, were widely distributed in floral nectar. We detected the highest concentration of these chemicals in *Citrus*, alongside the well-studied alkaloid caffeine. We explored the separate and interactive effects of these chemicals on insect pollinators in a series of behavioral experiments on bumblebees (*Bombus impatiens*). We found that octopamine and tyramine interacted with caffeine to alter key aspects of bee behavior relevant to plant fitness (sucrose responsiveness, long-term memory, and floral preferences). These results provide evidence for a means by which synergistic or antagonistic nectar chemistry might influence pollinators.

INTRODUCTION

Nectar is a chemically complex reward that modulates interactions between plants and their pollinators. Beyond sugar, nectar contains macronutrients (such as lipids and amino acids) and secondary compounds (such as alkaloids and phenolics) (Nicolson and Thornburg, 2007; Stevenson et al., 2017). These secondary compounds may be a byproduct of antiherbivore defenses in other plant tissue (Manson et al., 2012), but can also benefit the plant via their effects on pollinators, either by filtering the community of nectar consumers (Johnson et al., 2006) or by changing the behavior of visitors in a way that promotes plant reproduction (rev. Nicolson and Thornburg, 2007; Stevenson et al., 2017). For example, the pyridine alkaloid nicotine, found in *Nicotiana* nectar, is preferred at low concentrations by honeybees *Apis mellifera* (Singaravelan et al., 2005) and bumblebees *Bombus terrestris* (Baracchi et al., 2017), potentially boosting floral visitation and enhancing bees' learning and memory of these flowers. Similarly, caffeine, naturally found in the nectar of *Citrus* and *Coffea*, is preferred by honeybees at low concentrations (Singaravelan et al., 2005). Once consumed, caffeine enhances bees' memory of floral scent (Wright et al., 2013) and induces individuals to perceive nectar as higher quality than it is (Couvillon et al., 2015;Mustard, 2014).

Despite nectar's chemical diversity (Palmer-Young et al., 2018, 2019), our understanding of its effects on pollinators is limited in two key ways. First, though foliar chemical ecology highlights the relevance of synergistic effects (Richards et al., 2016), research on the behavioral effects of nectar chemistry typically involves a small number of phytochemicals studied in isolation (Adler, 2000; Stevenson et al., 2017), but see Estravis-Barcala et al., 2021; Hernández et al., 2018; Marchi et al., 2021; Richman et al., 2022; Thorburn et al., 2015). Secondly, though insect hormones and endocrine disruptors have long been identified in leaf tissues (Bowers, 1991), whether nectar is a site of any analogous chemical "cross-talk" between plants and pollinators (as explored for floral volatiles by Schiestl, 2010) is an obvious question (Mustard, 2020; Schultz and Appel, 2004). A number of chemicals that act on the insect nervous system have previously been discovered in nectar, including GABA, glutamate, and glycine, raising the question of whether plants may use neurotransmitters to manipulate pollinator behavior (Mustard, 2020; Nepi, 2014).

Intriguingly, in the decade after its discovery in octopus salivary glands (Roeder, 1999), the biogenic monoamine octopamine (**OA**) was identified in *Citrus* leaves and fruit (He et al., 2011; Stewart and Wheaton, 1964). As the invertebrate homologs of adrenaline and noradrenaline, OA and its biosynthetic precursor tyramine (**TA**) have broad roles as hormones and neurotransmitters (Roeder, 1999; Scheiner et al., 2006). Relevant to

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pollination, **OA** underlies the reinforcement pathways involved in associative learning in insects (Burke et al., 2012; Giurfa, 2006; Schwaerzel et al., 2003). For example, injections of **OA** into the brain of the honeybee can substitute for a reward in a learning paradigm (Hammer and Menzel, 1998). Sensory and cognitive neuroscientists have long noted that **OA** modulates bees' perception of reward quality, enhancing gustatory responsiveness and appetitive learning (rev. Scheiner et al., 2006). **TA**'s effects on reward perception are less well studied, but research to date indicates that it may have similar effects to **OA** on sucrose responsiveness and learning (Scheiner et al., 2017a, 2017b), while its effects on other behaviors may differ (*A. mellifera*: Schilcher et al., 2021; *Drosophila*: Saraswati et al., 2004). Although **OA** has been found to be present in leaves and fruit (He et al., 2011; Wheaton and Stewart, 1970), the possibility of it being present in floral nectar has never been explored. Combining analytical chemistry and behavioral assays on bumblebees, a model system for the study of cognition and pollinator behavior, we asked whether **OA** or **TA** was present in floral nectar at concentrations meaningful to insect pollinators.

Using liquid chromatography coupled to time-of-flight mass spectrometry (LC-TOF-MS), we searched for the biogenic amines **OA** and **TA** in nectar sampled from *Citrus* × *meyeri* plants, as well as from 14 additional species sampled opportunistically across 6 plant orders. Identities of biogenic amines in these samples were confirmed by comparison to authentic standards and doping (the addition of authentic **OA** standard to an analytical sample); Table S4. In *Citrus*, we found **OA** and **TA,** in addition to caffeine (**CA**), all at concentrations known to modulate bee behavior. Hence, we used concentrations within the range of those observed in the nectar of *C. meyeri*to ask how **OA** and **TA** affected aspects of bumblebee (*Bombus impatiens*) behavior, together and in concert with **CA**. Unlike previous work (Table 1), we did not assess the effects of **OA** and **TA** in isolation, since we always found them together in *Citrus* nectar, while **CA** presence varied. We examined the effects of these biogenic amines on four behaviors previously studied in the context of nectar chemistry (Si et al., 2005; Singaravelan et al., 2005; Thomson et al., 2015; Wright et al., 2013), with relevance for plant fitness (Burns, 2005; Cayenne Engel and Irwin, 2003): sucrose responsiveness, floral visitation rate, floral preferences, and early long-term memory performance.

RESULTS

Chemical analyses

We found **OA** was widely present in nectar samples extracted from single flowers across a broad range of plant taxa (Figure 1A). The highest concentrations were in *Citrus*, quantified for *C. meyeri* (N = 36 samples from 9 plants) at (mean ± SD): **OA**: 64.0 ± 73.3 μM (**TA**: 76.5 ± 71.8 μM), and *Citrus* × *paradisi* (N = 3 samples from 1 plant) at **OA**: 11.3 μM (**TA**: 16.9 μM). **CA** concentrations agreed with previous studies (Wright et al., 2013) and were only detected in *Citrus (C. meyeri*: **CA**: 28.5 ± 49.1 μM, *C. paradisi*: 23.6 μM). In *Citrus*, **OA** was highly correlated with **TA** (Figure 1B, $R^2 = 0.75$, $p < 0.001$) but in other species **TA** was only detected in *Daphne* and *Geranium*. While **TA** is a biosynthetic precursor to and should thus co-occur with **OA**, it had a higher limit of detection due to peak broadening. We could not identify the stereoisomers of **OA** and their relative ratios due to low concentration precluding the use of polarimetric or circular dichroism detectors (**OA** was not detected by our UV diode array detector). A high variance in **CA** presence was observed between *C. meyeri* individuals (Figure 1A). We also detected other known *Citrus* compounds in *C. meyeri* nectar, including phlorin and proline betaine, a proline derivative that may influence nectar preference (Carter et al., 2006).

Behavioral experiments

Results for experiment 1: Sucrose responsiveness

When we compared the responsiveness of bees pre-dosed with experimental nectar containing either 1) **OA** + **TA**, 2) **CA**, 3) **OA** + **TA** + **CA**, or 4) no compounds (control), an interactive effect emerged: while **CA** alone increased bees' tendency to extend their proboscis in response to antennal sucrose stimulation (**CA** vs. control: $z = -2.910$; $p = 0.019$; Figure 2A), this enhancement disappeared when bees were dosed with all three phytochemicals (**OA** + **TA** + **CA** vs. control: $z = -0.931$; $p = 0.788$; Figure 2A). On their own, **OA** + **TA** had no effect on sucrose responsiveness (**OA** + **TA** vs. control $z = -1.104$; $p = 0.687$; Figure 2A). As expected, bees were generally more responsive to higher concentrations of sucrose ($z = 7.092$; $p < 0.0001$).

Results for experiment 2: Floral visitation rate and preferences

Across the three different treatments, bees differed in the number of flowers they visited per second ($F_{2,37} = 6.521$, $p < 0.01$; Figure 2B), with individuals foraging the fastest in the **OA** + **TA** + **CA** treatment, and slowest

Table 1. Non-exhaustive summary of previous work addressing OA effects on bee behavior highlighting studies that used oral feeding over topical application and injection for more direct comparison to the current study

Study	Study species	Dosage of OA	Behavior measured	Outcome
Agarwal et al., (2011)	*Apis mellifera*	0, 0.25, 0.5, 1, and 2.5 mg/mL. Bees, on average, consumed 40 μL of this solution overnight	Spatial avoidance learning : bees shocked in a particular location w/color cue	OA-treated bees learned more slowly, i.e. negative effect on punishment learning. Effect reversed by antagonist
Barron et al., (2007)	*Apis mellifera*	Whole colonies treated by loading empty honeycomb with concentration of 10.5 mM	Dance behavior	OA increased the reporting of resource value in dances by forager bees
Cnaani et al., (2003)	*Bombus impatients*	0, 2, 5, and 8 mg/mL	Bees learn that a previously rewarding flower is no longer rewarding	OA did not affect flower choice, but affected the time interval between the change in reward status and the initiation of behavioral change in the bee
Mc Cabe et al., (2017)	*Melipona scutellaris*	0 μg; 9.5 μg (0.01 M); 19 μg OA (0.02 M); 38 μg OA (0.04 M).	Sucrose responsiveness	OA increased sucrose responsiveness
Muth et al. (2022)	*Bombus impatiens*	10 μL of 30% sucrose containing: 0 (control) 2 μg/μL or 8 μg/μL	Sucrose responsiveness Associative learning	OA increased sucrose responsiveness and conditioned response at highest dose
*Pankiw and Page (2003)	*Apis mellifera*	10 μL of 30% sucrose containing: 0 (control); *0.2 μg; 2.0 μg; or 20 μg	Sucrose responsiveness	OA increased sucrose responsiveness at all doses
Peng et al., (2020)	*Plebeia droryana*	Feeder of 0.01 M OA	Number of bees at a sucrose feeder with or without OA added	OA treatment caused a significant increase in the number of bees at artificial sucrose feeders and a 1.73 times higher individual foraging frequency
*Scheiner et al., (2002)	*Apis mellifera*	10 μL of 30% sucrose containing: 0.0 μg (control); *0.19 μg (10^{-4} M); 1.9 μg (10^{-3} M); or 9.0 μg (10^{-2} M).	Sucrose responsiveness	No effect at lowest OA dose; medium and high doses increased sucrose responsiveness
Schulz and Robinson (2001)	*Apis mellifera*	Fed colonies chronically with feeders of 2 mg mL^{-1}	Division of labor in honeybee colonies	OA treatment increased the number of foragers in a colony

Studies and doses marked with asterisks are most comparable to the doses used in the current study (see Table S1).

in the **OA + TA** treatment (Tukey post-hoc test controlling for multiple comparisons: **OA + TA** vs. **OA + TA + CA**: $t_{37} = -3.442$, $p = 0.004$; **CA** vs. **OA + TA**: $t_{37} = 2.047$, $p = 0.115$; **CA** vs. **OA + TA + CA**: $t_{37} = -1.221$, $p = 0.449$). The number of flowers visited per second did not differ across the sucrose control trials for these treatments ($F_{2,38} = 0.529$, $p = 0.583$). Bees assigned to different nectar treatments did not differ in the total number of flowers they visited, nor was their behavior in Trial 2 affected by the specific composition of the experimental nectar they had consumed in Trial 1. Bees were faster to visit flowers on their second trial than their first, but the strength of this effect did not vary across treatments (for all models and results see Table S5).

Preference test

When given a choice between the two flower types in the test trial, bees' preferences were driven by a combination of the experimental nectar and the color of flower (significant 2-way interactions between treatment × experimental nectar type, and treatment × color; Table S5). Bees generally had an aversion toward flowers with **CA** in their experimental nectar relative to control flowers (Figure 2C). However, this

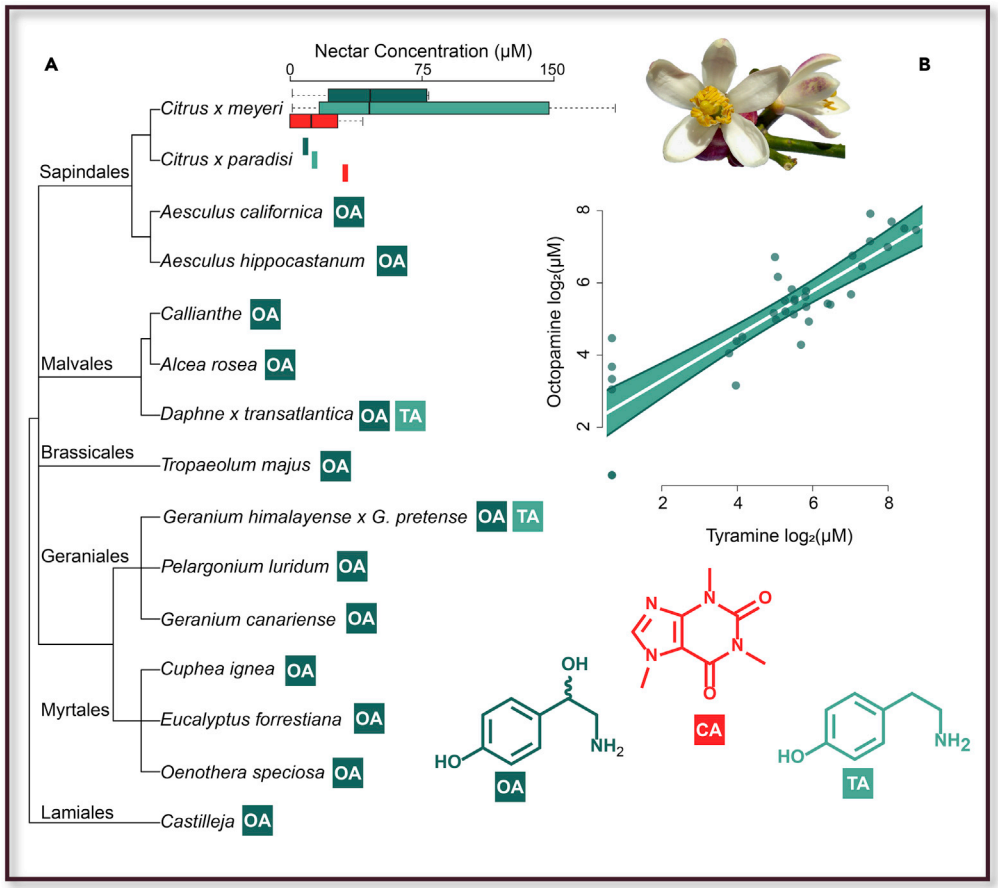

Figure 1. Biogenic amines in nectar

(A) **OA** was widely distributed across genera, with the highest concentration found in *Citrus*. Top left boxplot shows median, lower- and upper-quartiles, and whiskers 1.5× the interquartile range. Filled squares: **OA** and/or **TA** were detected in at least one sample.

(B) **OA** was correlated with **TA** in *Citrus* × *meyeri* nectar ($R^2 = 0.75$, $p < 0.001$); shading indicates 95% confidence intervals.

aversion was erased by the co-presence of **OA** + **TA** in the experimental nectar, which bees did not show a preference for or against on their own (**OA** + **TA** + **CA** vs. control: $z = -0.876$, $p = 0.381$; **OA** + **TA** vs. control: $z = -1.536$, $p = 0.125$; Figure 2C). Bees also had a strong preference for blue over yellow flowers, such that when the experimental nectar contained **CA**, bees discriminated more strongly against yellow flowers than blue (Figure S4).

Results for experiment 3: Long-term memory

We found a trend toward **CA** in experimental nectar enhancing bees' long-term memory of a visual association: 79.6% of bees fed **CA** during training remembered a color correctly the next day, compared to 63.6% of control bees ($z = 1.728$; $p = 0.084$; Figure 2D). However, any influence of **CA** on memory performance was erased by the co-presence of **OA** + **TA** in experimental nectar, which did not on their own affect performance (**OA** + **TA** vs. control: $z = 0.244$; $p = 0.807$; **OA** + **TA** + **CA** vs. control: $z = 0.364$, $p = 0.716$; Figure 2D). The color that the bee was trained to did not affect its long-term memory performance ($z = 1.268$; $p = 0.205$).

DISCUSSION

Octopamine (**OA**) plays an important role in the invertebrate nervous system as a neurohormone, neuromodulator, and neurotransmitter (Roeder, 1999). Here, we discovered **OA** present in floral nectar across 15 species and 6 orders of plants. Given that **OA** and its precursor tyramine (**TA**) orchestrate the metabolic, sensory, and cognitive basis of insect foraging, their presence in nectar suggests a new means of influence

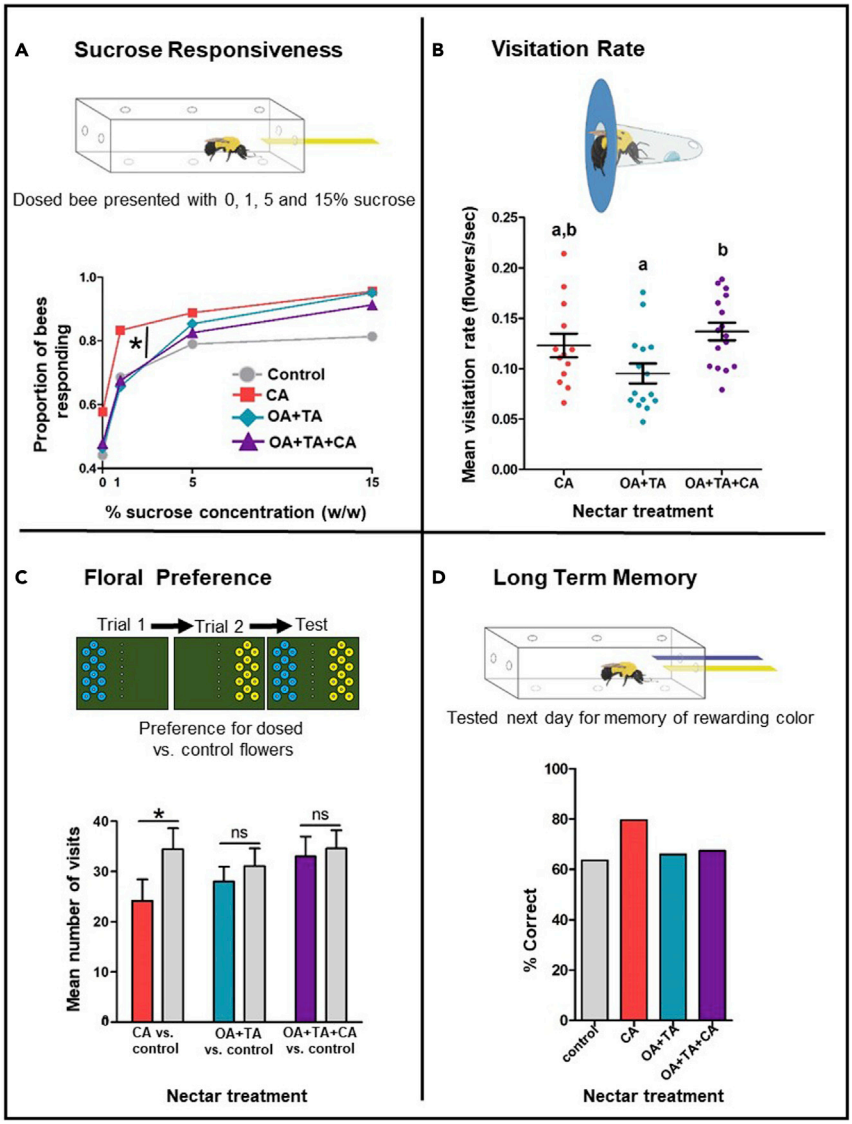

Figure 2. Secondary compounds identified in *C. meyeri* differentially affect bumble bee behavior

(A) CA in experimental nectar increased bees' sucrose responsiveness, an effect erased by the presence of OA + TA; asterisk indicates significance at $p < 0.05$ (GLMM, followed by Tukey post-hoc test).

(B) In a free-flying assay, bees visited artificial flowers fastest when they contained OA + TA + CA, and slowest when they contained OA + TA; letters on graph denote differences as determined by a Tukey post-hoc test; graph shows mean ±SEM.

(C) In a choice test, bees showed an aversion toward CA-containing flowers; this aversion disappeared when flowers' experimental nectar also contained OA + TA; graph shows mean ± SEM; asterisk indicates significance at $p < 0.05$ (GLMM, followed by Tukey post-hoc test).

(D) Bees trained to a visual association via absolute conditioning were tested the following day. The presence of CA in the experimental nectar trended toward enhancing memory ($p = 0.08$); this effect was erased when OA + TA were also present.

by which plants could manipulate the behavior of floral visitors by altering their perception of rewards and memory of floral signals. Yet, to date, all of what we know about these chemicals' effects on insect pollinators such as bees involves studying these compounds alone and at generally higher doses than we discovered in nectar (Table 1; Table S1). In combination, and at floral concentrations, we found that OA and TA did not have a direct effect on bumblebee behavior over the timescales most relevant to the movement of pollen. While many questions remain regarding how OA and TA might directly affect the behavior of other

floral visitors, our experiments show that for at least some bees, they can serve as potent modulators of other elements of nectar chemistry. This suggests a new perspective on the functional ecology of nectar traits, and highlights the need to consider the combinatorial effects of plant chemistry on the behavior of nectar consumers.

Far from a simple sugar solution, modern pollination biology emphasizes how nectar's secondary chemistry impacts the health and behavior of pollinators (rev. Mustard, 2020; Nepi, 2014), with implications for plant fitness. For example, the presence of alkaloids in floral nectar such as nicotine and **CA** has raised the intriguing prospect that plants might use nectar chemistry to alter the behavior of floral visitors to their own advantage. **CA**, for example, can enhance bees' long-term recall of floral scent (Wright et al., 2013), and we similarly noted a trend for **CA** to enhance bees' memory of a visual association. We also found that **CA** increased bees' gustatory responsiveness, making them more likely to extend their proboscis for a given concentration of sucrose. Increasing the perceived value of a given sucrose solution could explain how **CA** enhances bees' memory of a reward (Couvillon et al., 2015; Wright et al., 2013). We also found that free-flying bumblebees generally visited flowers containing **CA**-laced nectar at a faster rate, although this effect was largest (and only statistically significant) in the treatment that contained all three compounds. **CA** has been found to increase locomotor activity in a number of pollinators including flies, hornets, beetles, and bees (rev. Mustard, 2014). Our finding also points toward a potential benefit to the plant (Cayenne Engel and Irwin, 2003) in terms of increasing visitation frequency. **CA** in nectar thus has the potential to alter bee behavior in ways that might benefit a plant across multiple timescales via effects on the quality and quantity of pollination service—in theory, boosting bees' perception of nectar sugar content and making it more likely that they will remember floral features such as scent and color in the longer term.

Although the effects of **CA** on cognition are well established in this and other systems, concentration dependence is an important caveat: like most nectar constituents, **CA** itself is attractive or aversive depending on dose (Mustard et al., 2012). At the concentrations we found in *Citrus* x *meyeri*, bees had an aversion to **CA**, generally agreeing with previous findings. For example, Thomson et al. (2015) found indirect evidence for dose-dependent **CA** preferences in bumblebees: flowers with a low concentration of **CA** (0.01mM) received more pollen than flowers containing either a higher concentration (1mM) or no **CA** (Thomson et al., 2015). The concentration of **CA** in our free-flying experiment (0.04 mM) lies between the two concentrations of **CA** used in that study. This aversion is likely due to the taste of the **CA** in the flowers; honeybees find the taste of **CA** to be aversive, and bees are more sensitive to it via their proboscis than antennae (Mustard et al., 2012). However, despite this taste aversion, **CA** does not reduce honeybees' motivation to feed (Mustard et al., 2012), a result that we also saw here: bees that consumed **CA** did not have reduced feeding behavior in general, either in the total number of flowers they visited in Experiment 2 or in their sucrose responsiveness in Experiment 1.

Understanding the downstream effects of any one nectar chemical on multiple aspects of pollinator behavior relevant to plant fitness is a complex undertaking. Our findings here suggest that chemical context can be an important and often overlooked modulator of these effects. While the concentrations of **OA** and **TA** tested in our experiments did not alter bumblebee behavior on their own, we found intriguing interactive effects with **CA**. When **OA** and **TA** were present in the nectar solution, they eliminated **CA**'s effects on sucrose responsiveness, preferences, and (possibly) long-term memory. On its own, **CA** seems to carry several benefits for the plant: it may induce bees to accept lower quality nectar and improve recall of floral stimuli. Whether these behavioral effects would be enough to benefit the plant is an open question, especially since, at this concentration, **CA**-laden flowers might be avoided by bees. However, the presence of **OA** and **TA** alongside **CA** eliminates bees' aversion to **CA**-laden flowers, while enhancing one of its behavioral effects—a higher visitation rate, a finding that aligns with previous reports of **CA** increasing locomotor activity in insects (Mustard, 2014).

The costs and benefits of any one nectar secondary metabolite depend on ecological context (Gegear et al., 2007). Likewise, the interplay between the nectar constituents found here shows that phytochemical context can determine the pattern of downstream effects on bees. For humans, **CA** has behavioral effects that vary with phytochemical context (Schuster and Mitchell, 2019), but this has not been considered through an ecological lens: like most nectar secondary metabolites, **CA**'s effects on insects have

historically been studied in isolation (Baracchi et al., 2017; Thomson et al., 2015; Wright et al., 2013, but see Estravis-Barcala et al., 2021; Marchi et al., 2021). Methylxanthines such as **CA** can synergize with **OA** (Nathanson, 1984), potentially giving rise to the antagonistic effects observed here. Although **CA** and **OA** should work synergistically to increase cAMP accumulation through inhibition of phosphodiesterases and stimulation of adenyl cyclases respectively (Mustard, 2014), adenyl cyclase inhibition by **TA** (Blenau et al., 2000) may be responsible for mitigating these effects.

In contrast to previous work on the effects of **OA** and **TA** on bee behavior (summarized in Table 1, and in Muth et al., 2022), we did not find that these two compounds together affected sucrose responsiveness, memory, or foraging behavior. This discrepancy is likely due to dose and species differences. We detected a mean $\pm$ SD of **OA** in *C. meyeri* of 64.0 $\pm$ 73.3 μM, i.e. 9.8 ng in a 1 μL sample, which informed the doses we used in our behavioral experiments (see Table S1). Previous work using harnessed honeybees has found that **OA** increases sucrose responsiveness when individuals were fed doses that ranged from 0.2 to 20 μg (Pankiw and Page, 2003; Scheiner et al., 2002), i.e. the upper end of the doses we used and up to 100-fold higher. Replicating this work in bumblebees-fed doses of 20 and 80 μg, we found effects on sucrose responsiveness at the higher dose only (Muth et al., 2022). In the current study, we did not detect effects at the concentration found in *Citrus* (a dose of 0.24μg; see Table S1).

Previous work has identified other neurotransmitters in nectar, including GABA, glutamate, and glycine (rev. Mustard, 2020; Nepi, 2014). One study that addressed how GABA and beta-alanine affected bumblebees *B. terrestris* and honeybee *A. mellifera* behavior and survival found key differences between the species: above nectar-realistic levels enhanced survival only for bumblebees that also differed from honeybees in their concentration-dependent preferences for beta-alanine solution, and effects on motor behavior following consumption (Bogo et al., 2019). This further highlights the importance of not only using ecologically realistic nectar concentrations moving forward, but also considering the effects of chemicals on specific pollinator taxa. As highlighted in these reviews (Mustard, 2020; Nepi, 2014), we still know little in general about how nectar neurotransmitters influence pollinator behavior (but see Bogo et al., 2019; Carlesso et al., 2021; Felicioli et al., 2018; Inouye and Waller, 1984) and there is obvious scope for future work.

The adaptive significance of nectar secondary metabolites has been a longstanding question for pollination biologists (Adler, 2000). If their presence in nectar is an inevitable consequence of herbivore defense, beyond tissue-specific regulation (Manson et al., 2012), chemistry may be one strategy plants could use to fine-tune the effects of secondary metabolites on herbivores vs. pollinators. The widespread presence of foliar **OA** across plant taxa, from grasses (Hardwick and Axelrod, 1969) to bell peppers (Wheaton and Stewart, 1970), shows it is a basic part of many plants' biochemical toolkit. Currently, studies of fruit, leaf, and root chemistry are far more abundant than those of nectar. Subsequent examination of nectar chemistry in plants that produce known insect neurotransmitters (Mustard, 2020) could lead to unique insights into how tailored mixtures of phytochemicals influence pollinator behavior.

Limitations of the study

In the present study, we described the concentration of **OA**, **TA**, and **CA** in *Citrus* nectar and determined its effects on bumblebee behavior. Contrary to previous work, we did not find effects of **OA** and **TA** in isolation, but rather that they interacted with CA to alter key aspects of bee behavior. A clear next step would be to quantify **OA** and **TA** across a broader diversity of plants. Plant domestication may lead to differences in nectar chemical traits, although this has only been studied in a few cases (Egan et al., 2018; Palmer-Young et al., 2018, 2019). As such, it is possible that the concentration of **CA**, **OA**, and **TA** in *C. meyeri* may be less than in uncultivated Rutaceae, although it is worth noting that in the other plant taxa we sampled, concentrations were lower. A second area of future research would be to consider the role of these nectar constituents on different consumers. For example, beyond honeybees, **OA** has been shown to have similar effects on foraging and sucrose responsiveness in stingless bees *Plebeia droryana* and *Melipona scutellaris* (Mc Cabe et al., 2017; Peng et al., 2020), although at higher doses than detected in *Citrus* (Table 1). While bumblebees are commonly used as models to understand the effects of nectar chemicals on insect pollinators, species-specific responses to nectar chemistry (Bogo et al., 2019; Tiedeken et al., 2016) and agrochemicals (Cresswell et al., 2012; Piiroinen and Goulson, 2016) are known in other cases, and as such, pairing ecologically realistic levels of **OA** with co-occurring pollinators would allow for more precise evaluation of its function in nectar.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Chemical analyses
  - Behavioral experiments
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104765.

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AUTHOR CONTRIBUTIONS

A.S.L. conceived of the original question; C.S.P. conducted chemical analyses; F.M. conducted behavioral experiments. All authors provided expertise and feedback.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Optima Acetonitrile	Fisher Chemicals	Cat# A955-4; CAS:75-05-8
Optima Methanol	Fisher Chemicals	Cat# A456-4; CAS: 67-56-1
Optima Water	Fisher Chemicals	Cat# W64; CAS: 7732-18-5
LiChropur Formic acid	Supelco	Cat# 5330020050; CAS: 64-18-6
LiChropur Ammonium acetate	Supelco	Cat# 73594-25G-F; CAS: 631-61-8
(+/−)-Octopamine·HCl	AK Scientific, Inc.	Cat# M790-5g; CAS: 770-05-8
Tyramine	Alfa Aesar	Cat# J60990; CAS: 51-67-2
Caffeine	Sigma Aldrich	Cat# C5-3; CAS: 58-08-2
Experimental models: Organisms/strains		
Bumblebees	Koppert Biological Systems	https://www.koppertus.com/natupol-excel-start-up/
Software and algorithms		
MassHunter Qualitative Analysis	Agilent	https://www.agilent.com/en/product/software-informatics/mass-spectrometry-software
R statistical computing environment	R Project	https://www.r-project.org/
Solomon Coder	Solomon Coder	https://solomon.andraspeter.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by Felicity Muth (felicity.muth@austin.utexas.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data is uploaded as supplemental material and are publicly available as of the date of publication.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

For behavioral experiments, we used the bumble bee *Bombus impatiens* as a model to assess the effects of *Citrus* aminergic chemistry on foraging behavior. Across all experiments we used foraging workers from a total of 9 commercially-obtained colonies (Koppert Biological Systems, MI, USA), consisting of 50–100 workers at the start of the experiment, plus the natal queen. Colonies were maintained on sucrose solution (30% [w/w] unless stated otherwise), offered via a cotton-wicked feeder, and 1 tbsp honeybee-collected pollen (Koppert, USA), placed into the colony box every 2–3 days.

METHOD DETAILS

Chemical analyses

Sample and standard preparation

Citrus × *meyeri* samples were collected from established plants growing in residential gardens in Berkeley, CA (USA) or potted plants sourced from commercial nurseries. Other nectar samples were collected from

plants in gardens and field sites in Northern NV and Berkeley, CA as well as at the University of California, Berkeley Botanical Garden (Berkeley, CA). Nectar samples were collected using 1–5 μL microcapillary tubes (Drummond Scientific, USA) which were then photographed to estimate volume using ImageJ. Samples were pipetted onto filter paper for storage.

Filter paper-embedded nectar was dissolved with 3 × 0.5 mL of MeOH and dried in 2 mL autosampler vials. Dry nectar aliquots were reconstituted in 2 × 40 μL H_2O (80 μL total, Fisher, Optima) containing 0.1% formic acid (Supelco, LC-MS LiChropur). Standard stocks of **OA·HCl** (1.02 mg/mL **OA** equivalents, Acros), **TA** (1.31 mg/mL, Alfa-Aesar) and **CA** (1.31 mg/mL, Sigma-Aldrich) were prepared in 10 mL of H_2O (80 μL total, Fisher, Optima) containing 0.1% formic acid (Supelco, LC-MS LiChropur). Pooled stocks (100 μL each into 700 μL of 0.1% aq. formic acid) were diluted 100, 200, 400, 2000 and 4000-fold relative to the original stock concentration. On each day of analysis, 100 to 4000-fold dilutions were injected (20 μL) and subjected to analytical conditions described below.

LC-TOF analysis of nectar solutions

Chromatography was performed on an Agilent 1200 analytical HPLC equipped with a binary pump, autosampler, column compartment and diode array UV detector, coupled to an Agilent 6230 Time-of-Flight mass spectrometer via an electrospray ionization source (ESI-TOF; gas temperature: 325°C, flow: 8 L/m; nebulizer pressure: 35 psig; VCap: 3500 V; fragmentor: 125 V; skimmer: 65 V; octopole: 750 V). Analytical and standard solutions (20.00 μL) were injected and eluted at 0.4 mL/min through a Luna Omega Polar C18 column (Phenomenex, 2.1 × 100 mm, 2.6 μ, 100 Å) at 40°C. The linear binary gradient was comprised of buffers A: H_2O (Fisher, Optima) containing 10 mM ammonium acetate (Supelco, LC-MS LiChropur) and B: acetonitrile (Fisher, Optima) changing over 12 min accordingly: 0–2 min 0% B, ramp to 100% B at 5 min, hold at 100% B ramping to 0.8 mL/min 5-7 min, ramp to 0% B at 7.1 min, hold at 0% B ramping to 0.4 mL/min 7.1–12 min. **OA**, **TA**, and **CA** peak areas were extracted using the "find compounds by formula" function in Agilent MassHunter, including neutral losses of H_2O (**OA**) and NH_3 (**TA**). The identity of **OA** was confirmed by a doping experiment wherein a nectar sample solution prepared as above was mixed 1:1 with a 0.51 μg/mL solution of **OA** in 0.1% aq. formic acid. Nectar and doped nectar solutions were analyzed as described. Doped stock exhibited no peak broadening, but an increase in the **OA** peak area proportionate to doping was observed.

Standard curves

OA [retention time = 1.3 min; $[M + H]^+$ = 154.0856 (2.3%), $[M + H\text{-}H_2O]^+$ = 136.0758 (100%)], **TA** [retention time = 3.8 min; $[M + H]^+$ = 138.0913 (38%), $[M + H\text{-}NH_3]^+$ = 121.0650 (100%)] and **CA** [retention time = 6.0 min; $[M + H]^+$ = 195.0898 (100%)] were quantitated in nectar samples (Figure S3). Five-point standard curves were generated for **OA**, **TA** and **CA** (Table S4, Figure S2, top) at the beginning, middle and end of each chromatographic run. Each standard curve for each compound had an $R^2 > 0.98$. Within-day and between-day response errors are summarized in Table S4. Although within-day relative standard deviation (RSD) for **CA** standard curves was very high on day 2 (11.8%), which also affected between-day **CA** RSD (11.7%), **CA** was not detected in any of the samples analyzed on this day. Limit of quantitation (LOQ) was defined as the lowest concentration solution in the standard curve, and limit of detection (LOD) was defined as one-third the concentration of LOQ. Analysis concentrations below the LOD were considered not detected (ND), concentrations above the LOD but below the LOQ were defined as detected (D), and concentrations above the LOQ were used to calculate mean nectar concentrations (Table S4, Figure S3, bottom). Amine concentrations were converted from μg/mL to mM for publication. All analyses were carried out in R.

Behavioral experiments

Experimental nectar

In all experiments, we used the following concentrations dissolved in sucrose solution (hereafter referred to as 'experimental nectar'): **OA**: 8 μg/mL; **TA**: 10 μg/mL; **CA**: 6 μg/mL. These concentrations are within the range of the levels we discovered in *Citrus* × *meyeri* nectar (see Results) and when applicable we used realistic nectar volumes. Informed by our findings in *Citrus*, we always included both **OA** + **TA** together in solution, while **CA** presence varied. Table S1 summarizes how volume and timing of dosing varied across experiments; in all cases dosages used were on the lower end of those used in previous research where either **OA** or **TA** were (always separately) pharmacologically manipulated in other bee genera (Table 1).

Methods for experiment 1: Sucrose responsiveness. Phytochemicals that increase bees' gustatory responsiveness could elicit acceptance of lower quality nectar, reducing plants' total sugar production cost. **OA** and **TA** have generally been found to enhance bees' sucrose responsiveness (see Table 1 and [24, 25, 35]), but to gauge their combined effects at the concentrations found in *C. meyeri* nectar, we compared the tendency of workers that ingested the experimental nectars to show a proboscis extension response (PER) to sucrose solutions of increasing concentration.

We tested a total of 200 bees taken from 2 colonies (n = 10 bees in each of four treatments per day, i.e. 40 bees total per day). A colony box was connected via an entrance tunnel directly to a sucrose feeder; to collect motivated foragers for use in this experiment, we removed bees from this tunnel with an insect aspirator device. We cold-immobilized these bees before placing them into individual chambers (rectangular prisms sized l × w × h: 2.5 × 2.5 × 15cm; Figure 2A). Bees were given 1 h to acclimate to the chamber.

At the start of a trial, we dosed bees with a 30μL sucrose droplet (30% w/w) containing either a control nectar (sucrose only), or one of three experimental nectar solutions (**OA + TA**, **CA** or **OA + TA + CA**). Bees that that did not consume this droplet were excluded from the experiment (Control N = 3, **CA** N = 2, **OA + TA** N = 6, **OA + TA + CA** N = 4). Ten minutes after the bee had consumed the droplet, we offered it a strip of yellow card (Bazzill Cardstock, USA), which had been dipped into sucrose solution. The sucrose solution on the strip never contained **OA**, **TA**, or **CA**. The strip was presented to the bee's antennae for ~3 s. We offered a series of solutions in the same, ascending, order of sucrose concentration (0, 1, 5, 15% (w/w), with 3 s between each presentation and waited for the bee to retract its proboscis from the previous presentation before presenting it with the next one. We recorded if the bee extended its proboscis (i.e. exhibited a PER) to the solution or not as a measure of its responsiveness. The strip was always removed before the bee could contact it with its proboscis.

Experiment 1 (sucrose responsiveness) data analysis

To determine if a bees' tendency to respond to a given solution was affected by having previously consumed any of the experimental nectars, we carried out a binomial GLMM with the binary response variable responded/did not respond and the explanatory variables: treatment (control, **OA + TA**, **CA**, **OA + TA + CA**), the continuous variable 'solution concentration' and the random factors 'bee' and 'colony'.

Methods for experiment 2: Floral visitation rate and preferences. To explore the separate and combined effects of **OA, TA** and **CA** on floral choice, we conducted a free-flying foraging experiment. We tested 48 workers from 2 colonies (24 per colony; Table S2), maintained on 20% (w/w) sucrose pipetted directly into honeypots after each testing day, to minimize foraging experience outside of the experiment. We sequentially connected colonies to a foraging arena via a gated passageway, and trained bees to forage on vertical arrays of 12 and 24 artificial flowers (Figure S1).

Foraging arena and artificial flowers

The foraging arena (l × w × h: 1.23 × 0.6 × 0.6 m) was lit by a combination of LED (LED Wholesalers, Hayward CA) and full spectrum fluorescent lighting (True-Lite: F32T8-TL, Interlectric Corp., Warren, PA, 1100 lux in arena). The arena floor and back wall were painted green ("Ivy Topiary", Behr Ultra, Santa Ana CA). The back wall of the foraging arena had holes drilled into it, into which artificial flowers used in shaping, testing, and training (hereafter, "flowers") could be fitted.

Flowers (Figure 2B) were made from an Eppendorf tube (with a 4mm diameter hole cut in the end to allow sucrose to be pipetted in) surrounded by a 5cm corolla made from craft foam (Michaels, USA) that was either human-blue (i.e. blue as it is perceived by humans) or human-yellow (Figure S1).

Shaping

We 'shaped' bees, i.e. induced them to visit the experimental array via operant conditioning in a series of training steps. Initially, we gave a colony access to a human-white wicked feeder (20% w/w sucrose) in the foraging arena. After we observed around 20 foragers visiting the feeder, we then replaced the feeder with a shaping array. This array consisted of 8 artificial flowers (plastic Eppendorf tubes with the bottoms removed) situated in a vertical line in the center of the back wall; Figure S1). At first, each flower offered cotton soaked in 20% (w/w) sucrose protruding from the Eppendorf tube. As bees started to forage, we

then pulled the cotton into the tube, training bees to land and crawl into the flowers to gain sucrose rewards. When a bee did so, we paint-marked its thorax (with 2-3 colors of a range of possible colors) via a 5mm hole on the top of the Eppendorf tube. This allowed us to identify foragers who had experienced the shaping array. In the next shaping phase, we only allowed these marked foragers into the arena, where shaping flowers now offered a 2μL droplet of 20% sucrose solution. We noted which bees foraged on these flowers (via their individual paint-mark color combination) for use in the main experiment on the same day.

Training and testing

Shaped foragers were randomly assigned to one of three treatments, varying in the composition of experimental nectar offered by a given flower type. In all treatments, individual foragers encountered 12 of each of two flower types distinguished by color (blue or yellow), location (left or right), and by their nectar content: either experimental nectar containing the focal compound/s of interest (**CA, OA + TA,** or **CA + OA + TA)** in 30% (w/w) sucrose and control nectar containing 30% (w/w) sucrose solution without any compound present. We used both location and color cues to make as easy as possible for bees to learn the association between nectar type and flower type. During the trial, an experimenter standing behind the back wall of the arena refilled flowers (each containing 2μL solution) after the bee alighted on a subsequent flower.

All bees underwent two sampling trials to gain experience with each floral type (i.e. containing the nectar chemical or the sucrose-only control) sequentially and a final preference test where both flower types were offered simultaneously (i.e. 24 flowers in total). During all three visits to the arena, an individual forager was given free access to the array, and the trial ended when it returned to the colony or left the array for >2 min, in which case we returned it to the colony.

On Trial 1 the bee encountered a given nectar type (e.g. experimental nectar) in a particular flower type (e.g. blue) on a particular side of the arena (e.g. left). On Trial 2 they encountered the alternate nectar type (e.g. control) paired with the other color (e.g. yellow) on the other side of the arena (e.g. right). Thus bees could in principle use visual as well as spatial cues associated with each floral type. Whether the experimental nectar was offered on Trial 1 or 2, paired with the color blue or yellow, and positioned on the left or the right was balanced across experimental treatments and colonies. In the third (preference test) trial, the bee was presented with both flower types with the same location/color/reward pairing as during training.

To encourage the bee to start foraging at the start of Trial 1, if it did not enter a flower within 1 min, we transferred it to a flower using a plastic vial; bees' foraging was not interfered with after this point. To minimize variation in potential post-ingestive effects of experimental nectar on behavior, we used a gating system to control the timing of bees' re-entry to the foraging arena keeping it at a consistent 10 min inter-trial-interval. We wiped flowers between trials with 70% ethanol (for the corolla), and rinsed the Eppendorf tubes. At the end of the preference test, the bee was removed from the arena and euthanized.

Behavior coding

We filmed trials using an HD Sony camcorder (30 fps). From the videos we recorded each instance of a bee entering a flower to consume the nectar (Solomon Coder; https://solomoncoder.com) and thus obtained 1) the total number of floral visits bees made in each trial for each flower type and 2) how long bees spent foraging on each trial. From these data we described bees' foraging behavior in terms of their total number of flowers visited, their bout duration, visitation rate (number of flowers visited/bout duration), and preferences (relative number of each flower type visited on during the preference test). One recorded video (bee 31, trial 2) was corrupted so this behavioral data was not able to be included in the floral visitation rate analysis, but the third choice trial was included in the preference analysis.

Experiment 2 (floral visitation rate and preferences) data analysis

To determine if bees differed in their foraging behavior when encountering a single experimental nectar within a patch of flowers (i.e. in trials 1 and 2), we addressed: 1) the total number of flowers bees visited within a trial; 2) the rate at which bees visited flowers (total number of flowers visited within a trial/total foraging bout duration (secs)). For each of these, we ran models using just the trials were bees were given the experimental nectar of interest (i.e. no control trials), with the response variable being either ''number of flowers visited'' (using a GLMM with a Poisson distribution) or ''visitation rate'' (using a LMM) and the explanatory variables: experimental nectar (**OA + TA**, **CA** or **OA + TA + CA**), the trial order (1 or 2), the color

the solution was paired with (blue or yellow), the side the solution was on (left or right) and the random factors individual and colony. To assess the potential for any post-ingestive carryover effects on behavior, we additionally determined whether, when the trial with the control solution was on trial 2, if bees differed in their foraging behavior based on the experimental nectar that they had previously consumed on trial 1. We did this using the same response and explanatory variables described above.

To determine if bees preferred one flower type over the other, we compared the number of visits they made to each of the two flower types during trial 3. To do this, we carried out a GLMM with a Poisson distribution with the response variable being the number of flower visits to a particular flower type, and the explanatory variables treatment (**OA + TA**, **CA** or **OA + TA + CA**), solution type (experimental nectar or control), the order that the chosen solution type had been encountered (trial 1 or 2), the color of the chosen flower type (blue or yellow), the side of the chosen flower type (left or right), the order that the chosen color had been encountered (trials 1 or 2) and the random factors bee individual and colony. After we found no effect of the order on which the experimental compound had been experienced, we removed this factor from the model.

In all cases we ran full models initially, before removing non-significant interaction terms. When a model factor contained more than one level, we determined the significance of the term by running models with and without the factor of interest, and then compared models using the anova() function. Final models are shown in Table S5.

Methods for experiment 3: Long-term memory. To determine if the nectar compounds of interest affected long-term memory, we compared bees' performance at recalling a visual association learned using a modified version of the Free-Moving Proboscis Extension Response (FMPER) assay (Muth et al., 2017, see also Muth, 2021). In the protocol used here, individual workers learned to associate a sucrose reward with a colored strip of paper and were tested on their ability to recall this association a day later. We tested 204 bees (n = 5 colonies; Table S3) collected from colonies and placed into individual chambers as in Exp. 1.

Two hours after being placed in tubes, individual bees were trained via absolute conditioning over 5 trials spaced ~15–20 min apart. For each trial, the bee was presented with a given colored (yellow or blue) strip of card (Bazzill Cardstock, USA) dipped in 50% (w/w) sucrose containing either the nectar compound(s) of interest (**OA + TA**, **CA**, **OA + TA + CA**) or sucrose alone for the control group. Equal numbers of bees (10/treatment) were run for each of these four treatments on a given day. After the final trial, each individual bee was given an overnight feeder of 30% (w/w) sucrose.

The next day (20–23 h later), the feeders were removed and bee was tested for memory retention. To motivate bees, they were given a 10ul droplet of 50% (w/w) sucrose pipetted into their chamber. Five to ten minutes after the bee had consumed the droplet, it was presented with two unrewarding colored strips (blue and yellow), both dipped in water. The strips were inserted into the opposite end of the tube facing the bee (Figure 2D), such that the bee had to walk towards the strips and choose between the two; a choice was counted as a bee either antennating or extending its proboscis towards a strip. Twenty-one bees did not respond in the test phase, and were excluded from further analyses, resulting in the final sample sizes shown in Table S3.

Experiment 3 data analysis

To determine if a bees' long-term memory of a color was affected by the nectar type it had been trained with, we carried out a binomial GLM with the response variable correct/incorrect and the explanatory variables: treatment (control, **OA + TA**, **CA**, **OA + TA + CA**), color trained to (blue/yellow). "Colony" could not be included as a random factor in this case since it resulted in a singular fit error, however treatment sample sizes were evenly represented across colonies (Table S3).

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were conducted in R v. 4.0.5. We used the glmer() function in the lme4 package for GLMMs (Bates et al., 2015). To carry out post-hoc tests, we used the packages emmeans() (Lenth, 2017) and effects() (Fox and Weisberg, 2019). We included all key experimental factors in models as main effects (i.e. see Colegrave and Ruxton, 2017); interactions between model terms were included initially, but removed if non-significant. Details on the statistical analysis for each experiment are provided in each section above.