



Caffeine and ethanol in nectar interact with flower color impacting bumblebee behavior

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Abstract

Caffeine and ethanol are naturally occurring compounds in floral nectar. We examined how these compounds influenced pollinator behaviors including floral preference, floral constancy, and social behavior using bumblebees, *Bombus impatiens*, which were given prior experience foraging on either human blue or human white (hereafter blue and white) artificial flowers. Because flower color influenced bee behavior, with strong preferences for blue, we focused on the interaction between nectar chemistry and flower color. Bees that had experience with blue flowers preferred blue regardless of nectar chemistry. In contrast, for bees that had prior experience with white flowers, only the control treatment preferred white, while bees exposed to caffeine and ethanol showed no preference. The effects of nectar compounds may therefore only occur when bees are already foraging on a less-preferred color. We also examined the impact of nectar chemistry on the social behavior of joining other bees at flowers. In the same treatments for which bees showed a preference for previously experienced flower colors (all of the blue treatments and only the white control), bees also preferentially visited unoccupied flowers. In the treatments where bees showed no color preference, however (the white caffeine and ethanol treatments), bees showed no preference for unoccupied flowers. We show that the impacts of field-realistic levels of caffeine and ethanol in nectar on pollinator behavior depend on flower color, highlighting that the potential costs and benefits of nectar chemistry to plants are likely to be dependent on bee behavioral biases for other floral traits.

Significance statement

Flower nectar often contains toxic compounds hypothesized to impact pollination, but little research has shown their effects on the behavioral decisions of free-flying bees. Caffeine and alcohol occur in the nectar of some flowers. We found that bee response to these nectar compounds depends on the flower color. Bees preferentially visited blue flowers regardless of nectar chemistry, but the presence of caffeine or alcohol reduced bee color preference when bees had experience foraging on white flowers. The bumblebee's social behavior of joining other bees at flowers showed related effects; in treatments where bees showed a preference for flower type, they also preferred to forage alone. This research highlights that bees make decisions based on the interaction between multimodal cues including nectar chemistry, and therefore the strength of selection on nectar chemistry is dependent on bee behavioral biases for other floral traits.

Keywords Alcohol · *Bombus impatiens* · Bumblebee · Ethanol · Caffeine · Flower color · Foraging · Innate bias · Secondary metabolite · Social information

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Introduction

Pollinating insects make behavioral decisions about which flowers to visit using multimodal floral cues (Nordström et al. 2017), previous experience (Raine and Chittka 2008), and social information (Worden and Papaj 2005). This complex decision-making and consequences for plant pollination in tractable species have made pollinators a model system for cognitive ecology (Chittka et al. 2001; Leadbeater and

Chittka 2007). Pollen and nectar are the rewards pollinators associate with floral cues, but each of these resources contains its own chemistry (Stevenson 2020), which can influence pollinator preference and behavior (Pozo et al. 2014; Richardson et al. 2016). Floral nectar is a complex mixture of sugars, amino acids, plant secondary metabolites, and micro-organisms with their own metabolites (Percival 1961; Baker and Baker 1973; Vannette et al. 2013). While sugars and amino acids function as rewards for pollinators (Baker and Baker 1983, 1986), the function of plant secondary metabolites in nectar has remained less clear (Adler 2000; Stevenson 2020). One possibility is that plant secondary metabolites may prevent microbial degradation of nectar, including the build-up of microbial metabolites such as ethanol (González-Teuber and Heil 2009). A non-mutually exclusive alternative hypothesis is that plant secondary metabolites have positive effects on pollinators, for example, by changing pollinator behavior in ways that should be beneficial for plants, such as enhancing pollinator memory for floral odors (Wright et al. 2013). Finally, secondary compounds in nectar may have minimal effects on pollinators, but be expressed pleiotropically due to their function in other plant parts (Adler 2000). To better understand the costs and benefits of secondary metabolites in nectar, we examined the impacts on bumblebee behavior of two naturally occurring nectar compounds that we hypothesized would have opposing effects on bees: the plant secondary metabolite caffeine and the yeast metabolite ethanol.

Caffeine is hypothesized to have evolved as a deterrent to herbivorous insects (Nathanson 1984), and has been shown at high concentrations (0.01 M) to have negative effects on pollinator behavior (Mustard et al. 2012; Tiedeken et al. 2014). Caffeine, however, is present at low concentrations in the flowers of linden, citrus (10^{-5} M) and coffee (10^{-4} M) plants (Kretschmar and Baumann 1999; Naef et al. 2004; Maze et al. 2006; Wright et al. 2013) and at these concentrations causes nectar to be preferred by honeybees (Singaravelan et al. 2005), enhances honeybee learning and memory (Si et al. 2005; Wright et al. 2013), and increases bumblebee response to floral odors (Arnold et al. 2021). It has therefore been proposed that low concentrations of caffeine in nectar have positive repercussions for plant pollination, but this has not been studied directly. The most relevant study showed that the presence of caffeine in nectar at 10^{-5} M (but not 10^{-4} M) increased bumblebee deposition of artificial pollen (Thomson et al. 2015), but this study did not record individual bee visits or behavior; therefore, the mechanism for this increase in pollen deposition remains unknown. One way that caffeine in nectar may alter pollinator behavior to benefit plant pollination is by increasing bee floral preference or constancy. For example, nicotine, another alkaloid which acts on the same acetylcholine receptor as caffeine (Gauthier 2010), has been shown to increase preference, learning rate,

and constancy in *Bombus terrestris* (Baracchi et al. 2017). Floral preference is a measure of the proportion of visits to one flower type given its relative abundance, and floral constancy is a measure of the number of transitions between flowers of the same type versus different types (Gegear and Lavery 2005). Floral preference and constancy are both expected to affect how much conspecific versus heterospecific pollen bees are carrying (Waser 1983, 1986). Here we hypothesized that caffeine at the low-field-realistic dose present in *Citrus* sp. flowers (10^{-5} M) (Wright et al. 2013) would increase bumblebee preference for, and constancy to, the flower colors the caffeinated nectar was paired with.

We also studied how caffeine in flower nectar affects bumblebee social behavior. A body of work has emerged identifying the conditions under which bumblebees join other bees at flowers (Leadbeater and Chittka 2007; Leadbeater and Florent 2014; Jones et al. 2015; Dunlap et al. 2016; Smolla et al. 2016; Baracchi et al. 2018), but research has not yet examined how secondary metabolites in nectar affect this joining behavior. As joining other bees can be influenced by bee experience with flower quality or variability (Jones et al. 2015; Smolla et al. 2016), we were interested in whether nectar chemistry could impact joining behavior by altering bee perception of flower quality. Bumblebees are more likely to join other bees at flowers when they lack personal information, i.e., all the available flower options are novel to them (Leadbeater and Chittka 2007), or when their personal information is with low-quality flowers (Jones et al. 2015). Joining other bees, however, comes with risks in terms of competition for floral resources (Baude et al. 2011) or disease transmission (Figueroa et al. 2019). The social learning literature (Laland 2004; Kendal et al. 2018) therefore predicts that when bees have personal information about high-quality flowers, they should avoid competition from conspecifics, and this has been confirmed experimentally (Jones et al. 2015). Given that caffeine has been shown to increase preference and memory for flowers (Si et al. 2005; Wright et al. 2013; Arnold et al. 2021), one hypothesis is that the addition of caffeine to floral nectar would consequently reduce the propensity of bees to join other bees. Another possibility is that nectar chemistry impacts social behavior directly due to neuropharmacological changes (Brain et al. 1989; Mustard 2020). Studies have shown mixed effects of caffeine on bee social behaviors, including increasing social recruitment in honeybees (Couvillon et al. 2015), but no effect on social recruitment in stingless bees (Peng et al. 2019).

Floral nectar is frequently colonized by microbes including bacteria and yeasts in the field (Vannette et al. 2013; Pozo et al. 2014; Schaeffer et al. 2017; Vannette and Fukami 2017), and many of the yeasts present in floral nectar produce ethanol via fermentation (Ehlers and Olesen 1997; Lievens et al. 2015; Rering et al. 2018), although there is

little data available on how widespread ethanol is in flower nectar, or at what concentrations it most commonly occurs. Ethanol can occur in palm flower nectar at concentrations as high as 6.9% by volume (v/v) (Wiens et al. 2008; Hockings et al. 2015; Gochman et al. 2016). Low, likely to be field-realistic doses of 1% v/v ethanol cause free-flying honeybees to take longer to return to feeders (Bozic et al. 2006), and 2.5% v/v interfere with bee evaluation of floral reward quality (Abramson et al. 2005). Concentrations of ethanol equal to or greater than 5% impair honeybee learning (Abramson et al. 2000; Mustard et al. 2008). Given this previous research, we hypothesized that when ethanol nectar is paired with a flower color, bees may decrease preference for, and constancy to, that color. Another possibility is that if ethanol impairs bee behavioral decision-making, it could result in bees continuing to forage from inferior food sources rather than seeking out more rewarding food sources, resulting in higher constancy. If ethanol decreases preference for flowers, it could consequently increase the joining of other bees at flowers, as bees are more likely to join other bees when their experience is with low-quality flowers (Jones et al. 2015). An alternative hypothesis is that ethanol might directly impact social behavior in bumblebees, as has been shown with honeybees where 10% v/v ethanol decreased waggle dance behavior (Bozic et al. 2006) and reduced begging (Wright et al. 2012), and 2.5% causing bees to spend less time engaging in trophallaxis with other bees (Mixson et al. 2010).

Given the ubiquity of multimodal signaling in plant-pollinator interactions, and previous studies showing that bee response to nectar chemistry may depend on flower color (Muth et al. 2019), here we focus on the interaction of secondary metabolites and floral color, in our case human-perceived blue versus human-perceived white flowers. Given that *Bombus impatiens* typically show a preference for human-perceived blue over human-perceived white flowers (Simonds and Plowright 2004; Hudon and Plowright 2011), we expected bees to behave differently when foraging on these two flower colors, and studied how these differences interacted with nectar chemistry.

Materials and methods

We used 10 colonies of *Bombus impatiens* bumblebees purchased from Biobest (Biobest USA Inc. Leamington, Ontario). Colonies were provided with honeybee-collected pollen ad libitum and were tested under ambient light conditions in a greenhouse. Colonies were connected via a tube to a 114 cm × 69 cm × 30.5 cm plywood arena with a clear plexiglass top and side doors for access to the arena. The tube connecting the colony to the arena had sliding plexiglass doors to control entry and access to the arena. An entire

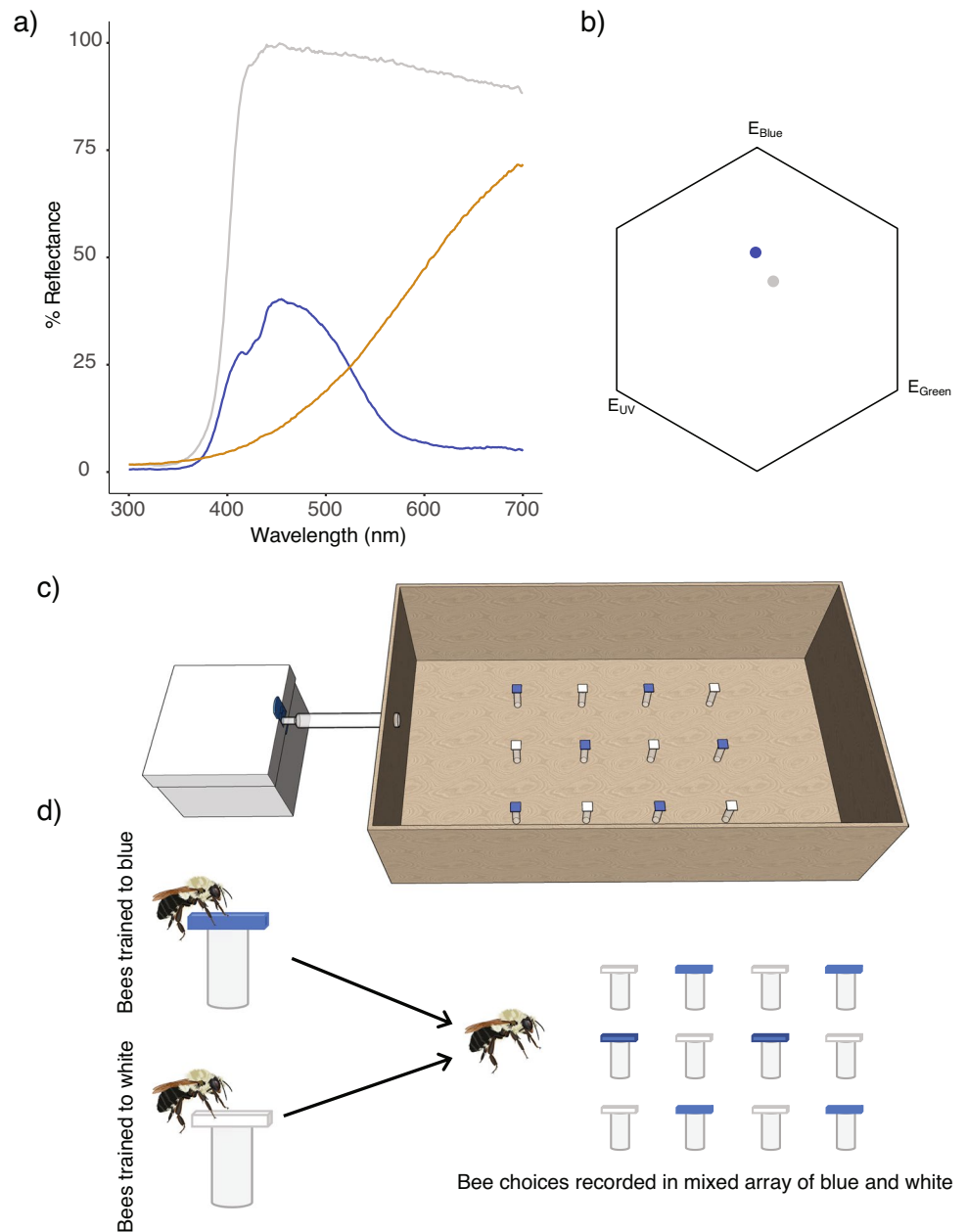
colony of bees were allowed to forage in the arena from clear acrylic square “flowers” (25 mm × 25 mm × 3 mm) on top of glass vials with 30% sucrose solution for at least 2 days for bees to learn to forage from artificial flowers in the arena. When bees were successfully foraging, they were all returned to their colony.

We used acrylic square (25 mm × 25 mm × 3 mm) “flowers” in two colors: human-perceived blue (Perspex Blue 727) and human-perceived white (Perspex White). To characterize these color stimuli, we measured their reflectance spectra and that of the plywood background in our arena using a Flame miniature spectrometer with a DH-2000 BAL UV-VIS-NIR light source and PTFE diffuse reflectance standard (Ocean Insight) (Fig. 1). We used the colourvision package in R (Gawryszewski 2018) to assess our stimuli using Chittka’s 1992 model of bee vision (Chittka 1992) with photoreceptor sensitivities of *Bombus impatiens* (Skorupski and Chittka 2010), and our reflectance spectra from the plywood of our arena as the background. The blue and white stimuli had a pairwise chromaticity distance of 0.21 hexagon units (hu) in *B. impatiens* color vision space (Fig. 1).

In the training phase, the arena was then set up with either 12 blue or 12 white flowers to let bees experience foraging on either blue or white. The flowers were provisioned with large volumes (~ 1 mL) of nectar solutions of either control (30% sucrose v/v = ~ 0.7 M sucrose), 10⁻⁵ M caffeine (in 0.7 M sucrose), or 0.17 M (1% v/v) EtOH (in 0.7 M sucrose). Each colony of bees received one nectar chemistry/flower color treatment. Each day of testing, the bees from the colony were allowed to forage in the arena for 2 h. Bees that were seen foraging were marked on the thorax with water-soluble fluorescent paint. After the 2 h, all bees in the arena were returned to the colony and the vials and arena floor were cleaned with 70% ethanol.

Marked bees (with paint demonstrating they had foraged in the 2-h experience phase) were then tested individually, with each bee randomly assigned to one of two experiments: a floral choice assay or a social behavior assay. The floral choice assay examined constancy to and preference for the flower color bees had previously experienced. For this assay, the arena was arranged with 6 white and 6 blue flowers, each provisioned with 15 µL of the nectar solution assigned to the colony (control, caffeine, or ethanol) (Fig. 1). The social behavior assay examined the degree to which bees joined other bees at flowers versus foraged solitarily. For the social assay, the arena was set up with 12 flowers of the color the colony had previous experience with (blue or white) with model demonstrator bees pinned to six of the flowers (Fig. 2). Demonstrator bees were worker individuals from other colonies frozen at -80 °C, pinned through the thorax with entomology pins, and then dried in a drying oven for 10 days at 60 °C to remove associated odors as used in similar bumblebee social behavior

Fig. 1 **a** Percent reflectance relative to PFTE diffuse reflectance standard for blue and white (blue and gray lines respectively) stimuli and plywood background (brown line). **b** Location of blue and white stimuli in the color vision space of *Bombus impatiens*. **c** Diagram to scale of setup with bumblebee colonies connected via a clear plastic tube to plywood foraging arenas. **d** Experimental design for floral choice assay where bees were given experience with either blue or white flowers and then foraged in a mixed array of blue and white flowers where we recorded their behavior. The same nectar chemistry treatment was applied to all the flowers in the array. Bumblebee illustration by Damond Kylo



experiments (Jones et al. 2015; Dunlap et al. 2016). Demonstrator bees were attached to flowers via their pins using white Fimo modeling clay, and clay was also attached to the alternative flower color to control for the presence of clay. The flowers were provisioned with 15 μ L of the nectar solution treatment assigned to that colony.

The floral choice and social behavior assays were video-recorded, and from the video (with the observer blind to nectar treatment) we determined the number of visits made to each flower type (color in the floral choice assay and occupied by a demonstrator bee or unoccupied in the social behavior assay). Flowers were refilled with 15 μ L of nectar solution when they were drained by a bee. Bees

were allowed to forage until they attempted to return to the colony or 5 min had passed. Bees that did not forage in 5 min were returned to the colony to be tested another time. Upon the completion of foraging, bees were captured and frozen to ensure they were not retested.

All statistics were conducted in R version 4.0.5. We used two measures of bee behavior to assess flower selection by bees in the floral choice assay experiment. First, we used an index of floral constancy calculated using Chittka's Index (Chittka et al. 2001; Arnold et al. 2021). This index is the proportion of transitions bees make between flowers of the same type versus flowers of different types. It is calculated as:

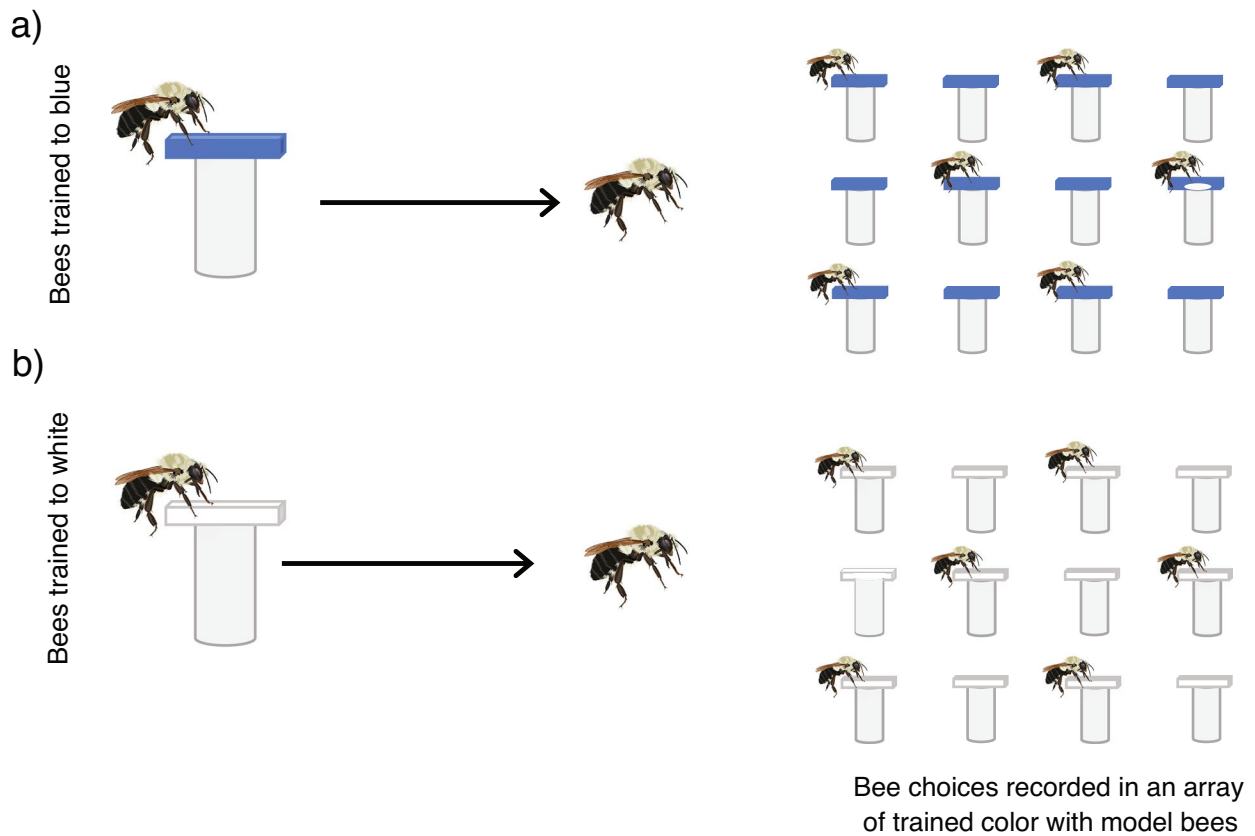


Fig. 2 Experimental design for the social behavior assay. Bees were given experience with either **a** blue or **b** white and then released to forage in an array of flowers of the color they had experience with

half of which were occupied by pinned demonstrator (model) bees. The same nectar chemistry treatment was applied to all the flowers in the array

$$\text{Chittka's Constancy Index} = 0.5[(A - B)/(A + B) + (C - D)/(C + D)]$$

where *A* is the number of transitions between white flowers, *B* is the number of transitions from white to blue flowers, *C* is transitions between blue flowers, and *D* is transitions from blue to white flowers. Chittka's Constancy Index ranges from 1 (constant to a flower type) to -1 (inconstant) with 0 as random foraging. We calculated Chittka's Index for each bee. We compared indices using a linear mixed-effects model using the lme4 package (Bates et al. 2015) with an interaction between nectar chemistry treatment and previously experienced color as a fixed effect and bee colony as a random effect. We conducted Tukey-corrected post hoc tests using the emmeans package (Lenth 2020). Within each treatment and experience color combination, we used a one-sample *t*-test to determine if bee scores for Chittka's index differed from 0 (random foraging). The second measure we calculated was Jacobs' Index of floral preference (Jacobs 1974; Gegeer and Laverly 2005). This is calculated as:

$$\text{Jacobs' Index} = (r - p)/(r + p - 2rp)$$

where *r* was the number of white flowers selected and *p* is 0.5, the proportion of white flowers available in the array. Values of Jacobs' Index ranged from 1 (preference for white) to -1 (preference for blue). We calculated Jacobs' Index for each bee. We compared indices using a linear mixed-effects model with the interaction between nectar chemistry treatment and previously experienced color as a fixed effect and colony as a random effect. We conducted Tukey-corrected post hoc tests. We used a one-sample *t*-test to determine if bee scores for Jacobs' Index differed from 0 (no color preference) within each combination of nectar chemistry and previously experienced color. In the social behavior assay, we analyzed the proportion of visits that bees made to occupied flowers using a linear mixed-effects model with the fixed effect of an interaction between nectar chemistry treatment and the color the bees had previous experience with and colony as a random effect. We used a one-sample *t*-test to determine if the proportion of visits bees made to occupied

flowers differed from 0.5 (no preference) within each combination of nectar chemistry and flower color.

Results

Floral choice assay

We tested 169 bumblebees from 10 colonies, with $N \geq 21$ bees per flower color \times nectar chemistry combination treatment. In the floral choice tests bees averaged 12.56 ± 6.37 SD flower visits. Floral constancy was affected by the previously experienced color (LMM; $\chi^2 = 37.10$, $df = 1$, $p < 0.001$), with higher constancy when bees had experience with human-perceived blue flowers. There was no overall effect of nectar chemistry treatment on constancy ($\chi^2 = 2.15$, $df = 2$, $p = 0.34$), but nectar treatment and previously experienced color showed an interaction ($\chi^2 = 6.04$, $df = 2$, $p = 0.049$). The random effect of colony in the model had an intercept variance < 0.001 , indicating that colony identity did not affect bee floral constancy. Tukey-corrected post hoc tests between treatments showed that bees that experienced caffeinated blue flowers were more constant than bees that experienced control white flowers ($p = 0.044$), but the reverse comparison was not statistically significant ($P = 0.16$). To determine whether bees were constant for each treatment, we compared constancy index scores to 0 (random foraging). Bees with experience with blue flowers were constant regardless of nectar chemistry (one-sample t -test: control $t = 5.20$, $df = 32$, $p < 0.001$, caffeine $t = 5.43$, $df = 30$, $p < 0.001$, ethanol $t = 2.32$, $df = 39$, $p = 0.03$; Fig. 3a). Conversely, none of treatments with experience with white had constancy scores statistically different from 0, indicating bees in these treatments were foraging randomly.

We also analyzed bee choices using Jacobs' Index for floral preference (Jacobs 1974; Gegear and Laverty 2005; Austin et al. 2019) which assesses selection of a particular flower type given its relative abundance. Floral preference depended on an interaction between nectar treatment and previously experienced color ($\chi^2 = 9.71$, $df = 2$, $p < 0.01$; main effect of previously experienced color LMM; $\chi^2 = 91.59$, $df = 1$, $p < 0.001$, main effect of nectar treatment $\chi^2 = 3.17$, $df = 2$, $p = 0.20$). The random effect of colony in the model had an intercept variance < 0.001 , indicating that colony identity did not affect bee floral preference. Tukey-corrected post hoc tests indicated that bees with experience with white in the control nectar treatment had different preferences than bees with experience with blue for the control ($p < 0.01$), caffeine ($p < 0.01$), and ethanol ($p = 0.02$) treatments. When we compared preference scores for each treatment to 0 (no preference), bees that had experience with blue all showed a preference for blue (one-sample t -test: control $t = -11.22$, $df = 32$, $p < 0.001$, caffeine $t = -6.51$, $df = 30$, $p < 0.001$, ethanol $t = -5.67$, $df = 39$, $p < 0.001$; Fig. 3b). Of the bees with

experience with white, only the control treatment showed a preference for white ($t = 3.82$, $df = 22$, $p < 0.001$), while the caffeine and ethanol treatments showed no preference.

Social behavior assay

We tested 174 different bees from the same 10 colonies in the social behavior assay, with $N \geq 21$ bees per flower color \times nectar chemistry combination treatment. We recorded bee visits to flowers occupied by model bees and unoccupied flowers. There was no overall effect of nectar chemistry (LMM; $\chi^2 = 3.41$, $df = 2$, $p = 0.18$) on joining behavior by bees. The flower color marginally, but not significantly, affected joining behavior ($\chi^2 = 2.85$, $df = 1$, $p = 0.092$), with more visits to occupied flowers when bees were foraging on the less-preferred white flowers. There was no interaction between nectar chemistry and flower color ($\chi^2 = 0.66$, $df = 2$, $p = 0.72$) on the proportion of visits bees made to occupied flowers. The random effect of colony had an intercept variance of 0.0019 and standard deviation of 0.043 indicating that colony identity had a small effect on social behavior. When we compared the proportion of visits that bees made to occupied versus unoccupied flowers to 0.5 (no preference), bees foraging on blue flowers all showed a significant avoidance of occupied flowers (one-sample t -test: control $t = -2.34$, $df = 32$, $p = 0.026$, caffeine $t = -6.07$, $df = 35$, $p < 0.001$, ethanol $t = -3.60$, $df = 40$, $p < 0.001$; Fig. 3c). Of the bees with experience with white, only the control treatment avoided occupied flowers ($t = -2.23$, $df = 21$, $p = 0.037$), while the caffeine treatment showed a marginal avoidance effect ($t = -2.08$, $df = 20$, $P = 0.051$) and the ethanol treatment showed no preference ($t = -0.33$, $df = 20$, $P = 0.74$).

Discussion

Flowers are multimodal displays, presenting pollinators with a range of cues for which they have innate and learned preferences (Kulahci et al. 2008; Austin et al. 2019). Bee's preference for flower color in a mixed array interacted with nectar chemistry and was different between the two colors our bees had experienced. Caffeine, a plant secondary metabolite, and ethanol, a yeast metabolite, erased bee floral preference, but these effects were mild and largely outweighed by strong effects of flower color. Bees with previous experience with human-perceived blue preferred blue in mixed arrays regardless of nectar chemistry, whereas bees with experience with human-perceived white and consuming ethanol and caffeine showed no preference. Preference, therefore, demonstrates an interaction between experienced color and realistic nectar chemistry, with the effects of chemistry only seen for the less-preferred color. A similar phenomenon has been shown in an

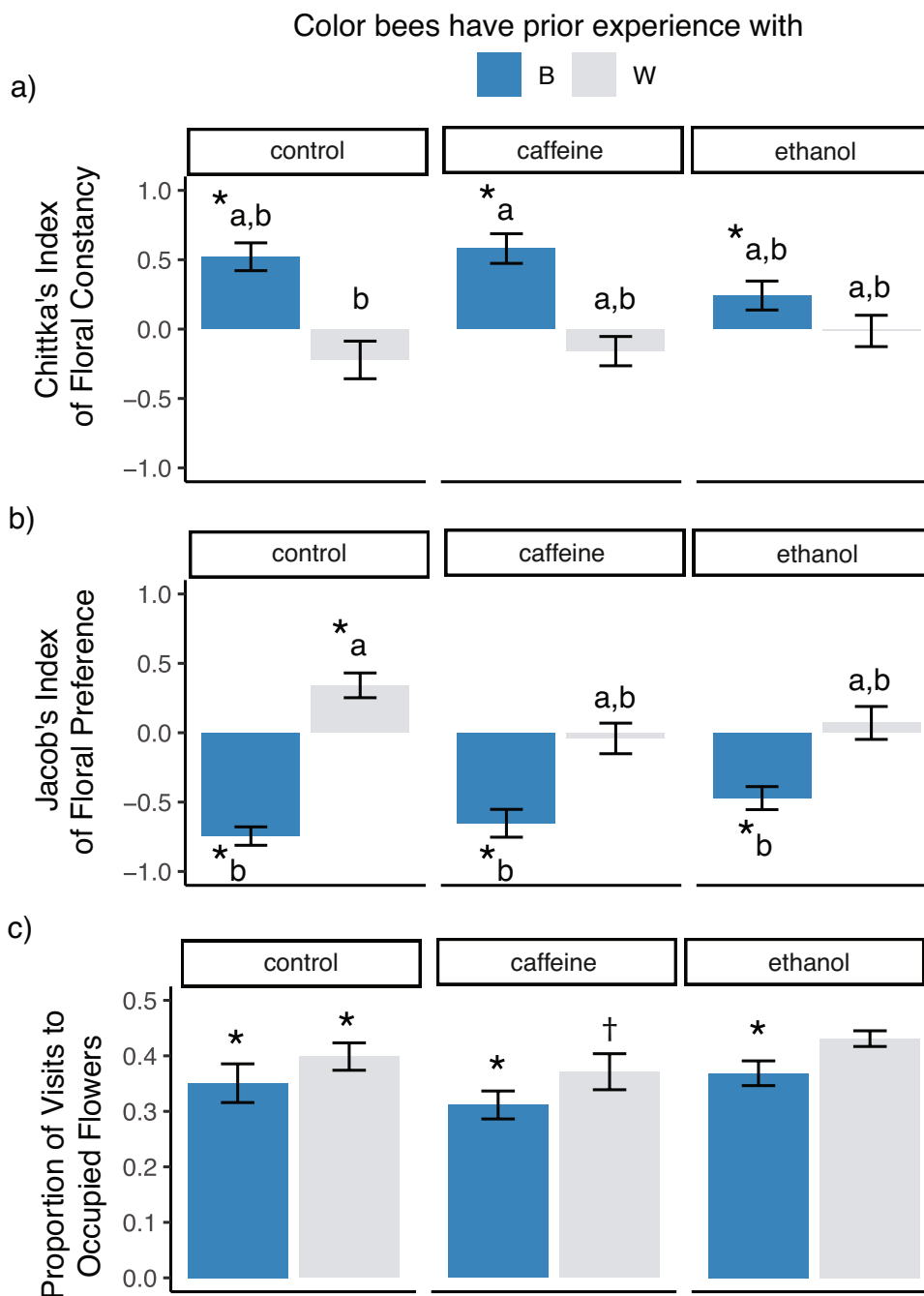


Fig. 3 Bee experience with color interacts with nectar chemistry for behavior. **a** Chittka's Index of floral constancy ranges from 1 (constant) to -1 (inconstant) with 0 as random foraging. Blue bars are means and standard errors of bees with previous experience with blue, and gray bars are means and standard errors of bees with previous experience with white. Asterisks indicate significant ($p < 0.05$) differences from 0 (random foraging). Different letters indicate significant differences between treatments according to Tukey-corrected post hoc tests. **b** Jacobs' Index of floral preference ranges from 1 (preference for white) to -1 (preference for blue). Blue bars are means and standard errors of bees with previous experience with

blue, and gray bars are means and standard errors of bees with previous experience with white. Asterisks indicate significant ($p < 0.05$) differences from 0 (no preference). Different letters indicate significant differences between treatments according to Tukey-corrected post hoc tests. **c** Proportion of visits made by bees to occupied flowers in each nectar chemistry and flower color treatment. Asterisks indicate significant ($p < 0.05$) differences from 0.5 (no preference), and the cross indicates a marginal difference ($p = 0.051$). Different letters indicate significant differences between treatments according to Tukey-corrected post hoc tests

experiment with neonicotinoid pesticides and bumblebees, where the effect of the pesticide on learning errors was dependent on the color or odor pairing during training (Muth et al. 2019). Context-dependent effects on bumblebees have also been shown for nectar alkaloids (Gegear et al. 2007). For our bees, it appears that the impact of realistic levels of caffeine and ethanol is dependent on associated floral cues. The consequence for plants could be that the presence of plant secondary metabolites like caffeine with direct effects on behavior, or plant secondary metabolites with antimicrobial effects that prevent the accumulation of ethanol, could have costs and benefits that are dependent on other floral traits.

Overall, we found that bees showed higher preference and constancy to human-perceived blue flowers over human-perceived white flowers in our experiments. A preference for blue over white has been shown previously for *Bombus impatiens* (Simonds and Plowright 2004; Hudon and Plowright 2011), as well as for honeybees, *Apis mellifera* (Giurfa et al. 1995). In the UK, innate preferences for blue and violet flowers in *Bombus terrestris* have been proposed to be adaptive in the field due to higher nectar volumes in blue and violet flowers than in other available colors (Raine and Chittka 2007). One possibility, therefore, is that the behavioral differences we see in our bees in response to colors are due to an innate preference for blue that has been adaptive over their evolutionary history. Another possibility, however, is that the white flowers were more difficult for bees to distinguish from the background (Kevan et al. 1996). Our spectral measurements indicated that our two stimuli were separated by 0.21 hexagon units (hu) in chromaticity distance of *Bombus impatiens* color vision space and given our wood background. As our bees showed preferences for white when they had experience with white in the control treatments, it is clear they were able to distinguish the white flowers from the background as well as from the blue flowers. It is possible, however, that the lack of preference we see in the caffeine and ethanol treatments is due to an impaired ability to distinguish white flowers from the background rather than avoidance of white flowers. Although not statistically different, preference for blue flowers also declined with the presence of caffeine and ethanol in nectar, indicating that it may not be a different mechanism occurring with white flowers, just a stronger effect. It could be worthwhile to repeat these experiments with other color pairings.

Caffeine is present in the flowers of several species, across at least three botanical families (Wright et al. 2013) and has been shown under some conditions to enhance honeybee learning, memory (Si et al. 2005; Wright et al. 2013), and social recruitment behavior (Couvillon et al. 2015). The presence of caffeine at 10^{-5} M can also increase bumblebee deposition of artificial pollen (Thomson et al. 2015), and at 10^{-4} M improves memory for floral odor (Arnold et al.

2021). Our bees experiencing 10^{-5} M caffeine (what occurs in citrus nectar) in the nectar of white flowers showed no color preference in mixed arrays unlike the sucrose control on white flowers, or bees experiencing caffeine on blue flowers. Some studies have similarly shown negative effects of caffeine on behavior such as decreasing the responsiveness of honeybees in olfactory learning trials, and reducing learning performance (Mustard et al. 2012), and caffeine is believed to have evolved as a deterrent to feeding insects (Nathanson 1984). A study with Brazilian populations of the stingless bee *Plebeia droryana*, which have co-evolved with plants in the *Citrus* and *Coffea* genera, shows no effect of caffeine on either foraging or social recruitment behaviors (Peng et al. 2019), indicating the potential for co-evolved pollinators to be resistant to the neuropharmacological effects of caffeine. As the majority of research showing positive impacts of caffeine on pollinator behavior have used olfactory learning, it could be that the ways that caffeine affect bee behavior are different between olfactory and visual floral cues. It should be noted, however, that nicotine, an alkaloid with similar neuropharmacological action to caffeine, has been shown to affect bumblebee behavioral responses to visual stimuli at natural concentrations (Baracchi et al. 2017).

Flower nectar containing ethanol is known to be consumed by pollinators (Kevan et al. 1988; Ehlers and Olesen 1997; Wiens et al. 2008; Beaulieu et al. 2017). While there is little available data on ethanol levels in flowers, we used 0.17 M (1% v/v) which we expected to be realistic as a low-field concentration. Similar to caffeine, bees with prior experience with ethanol in nectar on white flowers showed no color preference in a mixed array, whereas bees with prior experience with ethanol in nectar on blue flowers did show a preference for blue (although a reduced preference). This result would indicate that ethanol may have aversive effects on bumblebees, as has been shown in other studies with honeybees (Abramson et al. 2000, 2015), including in doses as low as 1% v/v (Bozic et al. 2006), but this effect was more evident in our study when paired with the less-preferred color.

Bee's response to nectar chemistry also differed between flower colors in the social behavior assay. There was an overall trend towards bees making more visits to occupied flowers when they were foraging on white flowers than blue flowers, and in all of our treatments where bees were foraging on blue flowers, they showed a significant preference for unoccupied flowers. Given that bees preferentially visited blue flowers in the floral choice assay, this result is supportive of the literature which has shown that when bees forage on high-quality flowers, or consistently rewarded flowers, they are less likely to join other bees (Jones et al. 2015; Smolla et al. 2016). In our case, it was color preferences that influenced bee joining behavior rather than rewards, but color biases have also been previously shown to influence social behavior (Jones

et al. 2015). Both caffeine (Couvillon et al. 2015) and ethanol (Bozic et al. 2006; Mixson et al. 2010; Wright et al. 2012) have been shown to affect social behaviors in honeybees. In our study when ethanol (and marginally caffeine) was present and bees were foraging on less-preferred white flowers, bees did not show the preference for unoccupied flowers we saw in the other treatments. This result is concordant with results from the floral choice assay, as these are the treatments in which bees showed no preference for white flowers, even though these are different individual bees. The social behavior assay therefore supports our data showing subtle effects of caffeine and ethanol when paired with white flowers, in this case resulting in bees being more likely to join other bees in these treatments than in other nectar chemistry and flower color treatments. It is important to note, however, that we found colony variation in bee social behavior and therefore bee social response to nectar chemistry and flower color may differ by colony. This result points to an indirect effect of caffeine and ethanol on joining behavior through changes in perception of floral quality rather than a direct neuropharmacological effect on social behavior which we might expect to change behavior across all chemistry treatments instead of being dependent on flower color.

Pollinator behavior on flowers is determined by a complex combination of the multimodal cues of the flowers themselves (Nordström et al. 2017), the associated rewards the plant provides to pollinators (Hazlehurst and Karubian 2016), the pollinator's innate biases (Schiestl and Johnson 2013) and previous experience (Ferdy et al. 1998), the social context in which pollinators forage (Baude et al. 2008), and chemistry of floral nectar (Wright et al. 2013). These different components influencing pollination are usually studied in isolation. In studying their interaction, we demonstrate the context-dependent responsiveness of bees to caffeine and ethanol in nectar, as well as potential evolutionary constraints on the costs and benefits of secondary metabolites in flower nectar based on flower color. To maintain ecological relevance, we used concentrations of compounds that are on the low end of the range seen in the field. Our results indicate that while these low natural concentrations can influence bee behavior, their effects are heavily influenced by biases for flower color, highlighting the importance of studying interactions between floral stimuli and nectar chemistry.

Despite the multimodal nature of flowers, a strong attractive signal in one modality can drive overall bee preferences. However, under most natural conditions, with multiple floral colors and odors, and variable nectar chemistry, it is the sum or interaction of these factors that will dictate bee behavior. From a plant's perspective, multimodal signaling may be critical both because of variation in pollinator species and in the surrounding plant community. Regardless, it is increasingly clear that interactive effects on bee behavior are common, and studying their consequences is an important future direction.

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Data availability All data generated in this study are included in the supplementary materials.

Declarations

Conflict of interest The authors declare no competing interests.

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