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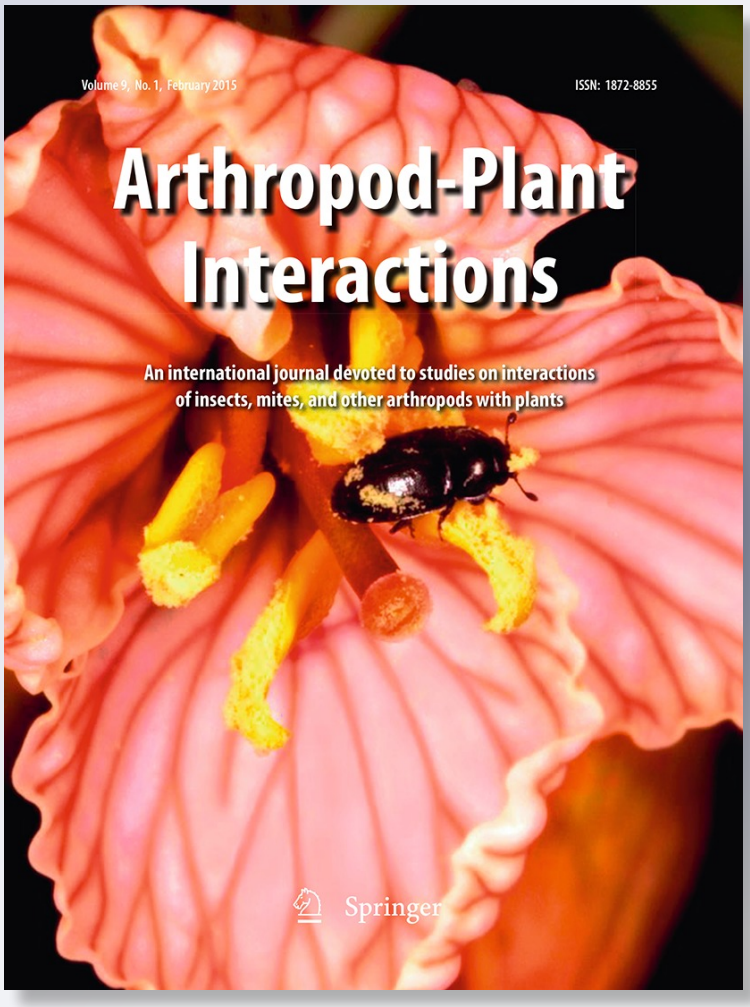

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ORIGINAL PAPER

Flowers with caffeinated nectar receive more pollination

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Abstract Floral nectar functions to attract insects, so the inclusion of toxic compounds calls for explanation. Recent work shows that honeybees prefer nectars with low concentrations of caffeine and nicotine, and that associative learning by honeybees is enhanced by caffeine, prompting speculation that pollination service could be enhanced. We directly tested caffeine's effect on pollination service by allowing bumblebee colonies to feed on arrays of artificial flowers that offer nectar while also dispensing and receiving dye particles as pollen analogues. With caffeine levels signaled by flower color (blue, green, or yellow) in a factorial design, flowers offering nectar with 10^{-5} M caffeine received significantly more pollen analogue than did those with 10^{-4} M caffeine or with no caffeine. Effects of caffeine were unaffected by which colors were associated with which caffeine levels: Color alone had no significant effect, and there was no interaction between color and caffeine level. In cases where greater pollination service translates to increased fitness, we would expect stabilizing selection to maintain nectar caffeine at intermediate levels.

Keywords Floral nectar · Caffeine · Pollination · Bumblebee · Artificial flower · Secondary compound · Addiction

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Introduction

Floral nectars frequently contain low concentrations of phenolics, alkaloids, and other potent secondary metabolites that are generally toxic at high enough doses. When such compounds occur in other tissues such as leaves, their function is usually thought to be deterrence of herbivores; therefore, their presence in a presumably attractive substance like nectar seems paradoxical and has prompted numerous adaptive hypotheses over recent decades [e.g., Rhoades and Bergdahl 1981; review by Adler (2000), Tiedeken et al. (2014)]. To date, tests of these hypotheses have provided mixed results, and no consistent conclusion regarding the responses of flower-feeding insects to secondary metabolites. Alkaloids certainly can induce aversion by bees, but the effects depend on dosage and ecological circumstances. Demonstrations of deterrence are balanced by demonstrations of no effect. Detzel and Wink (1993) reported that large groups of captive honeybees (*Apis mellifera*) avoided 18 of 19 alkaloids tested (including caffeine), at a range of concentrations, in pairwise choice tests against alkaloid-free sugar solutions. Quinine has frequently been used for aversive conditioning in experiments with bumblebees (recent review by Rodríguez-Gironés et al. (2013)). Adler and Irwin (2012) reported that flowers of *Gelsemium sempervirens* with artificially elevated levels of the alkaloid gelsemine received less pollen than did those with artificially reduced gelsemine. Analogously, Gegear et al. (2007) showed that captive bumblebees avoided artificial flowers with gelsemine-laced sucrose in favor of equally concentrated sucrose solution, but that their aversion turned to preference if the gelsemine solutions offered higher sugar concentrations. Manson et al. (2013a) also showed that norditerpene alkaloids in *Delphinium barbeyi* nectar

deterred bumblebees, but only at concentrations much higher than those found in nature. For *Apis* and *Bombus*, at least, it seems possible that repellent effects of nectar alkaloids might frequently be negligible at natural concentrations, a point strongly emphasized by Tiedeken et al. (2014) based on preference tests using the bumblebee *Bombus terrestris dalmatinus*. It remains possible that "repellent" chemicals turn up in nectar most often through diffusive "leakage" from other tissues in which their repellent role is preserved by selection, i.e., their presence *in nectar* is essentially a non-adaptive consequence of their occurrence in a plant's total suite of metabolites (Manson et al. 2013b).

In contrast to the general tendency for nectar alkaloids to range from aversive to neutral, Singaravelan et al. (2005) offered the alternative hypothesis that low concentrations of psychoactive alkaloids might actually be attractive, as if by rendering floral nectar "addictive" to flower visitors. They showed that free-foraging *A. mellifera* workers took more nectar from open feeders when the solutions contained caffeine or nicotine. That result received impetus recently from the demonstration that captive honeybees (*A. mellifera*) showed a stronger proboscis extension reflex (PER) to a scent cue if they had previously received both sugar and caffeine along with the scent, rather than sugar without caffeine (Wright et al. 2013). Wright et al. also presented data suggesting that honeybees were more likely to reject sugar solutions at higher concentrations of caffeine, introducing some confusion. Tiedeken et al. (2014) cite Hagler and Buchmann (1993) as reporting *A. mellifera* to prefer low concentrations of caffeine, but Hagler and Buchmann actually tested caffeic acid, an unrelated compound). Chittka and Peng (2013) point out that "addiction" is an onerous term. It implies persistent pursuit of an activity that damages the pursuer and induces withdrawal effects. Without evidence of such damage, it is better to speak of simple preference rather than addiction.

What are the consequences of caffeinated nectar for pollination service? Singaravelan et al. (2005) suggested that bee preferences might lead to better pollination through a "pollinator fidelity" mechanism proposed by Baker and Baker (1975); see Adler (2000). That idea rests on selective attraction of better pollinators and deterrence of worse ones, but the hypothesis is not developed in detail. Singaravelan et al. (2005) call for further study: "Conceivably, a considerable number of alkaloids in nectar (e.g., nicotine, caffeine, cannabinoids) have both addictive and aversive properties and have not yet been studied in an ecological context." Wright et al. (2013) presented a hypothetical scenario by which caffeine's role in strengthening associative learning might translate to better pollination service for caffeine-presenting flowers, but they did not test its premises.

Here, we present a more direct test of the effects of caffeine-laced nectar on pollination. Because manipulative experiments on nectar chemistry would be logistically demanding in natural plant communities, we used a physical model system of artificial flowers to estimate pollination success directly. The system measures the amounts of a pollen analogue transferred among arrays of flowers visited by colonies of bumblebees (*B. impatiens*) foraging freely in large indoor flight cages. Using artificial flowers allows us to simultaneously compare pollen receipt among floral phenotypes that vary in caffeine concentration. Permitting numerous worker bees from several colonies to visit simultaneously, and for long periods, allows our experiments to include realistic effects such as bee–bee competition and the possible development of ideal-free distributions of foraging effort across floral phenotypes. Such higher-level behavioral effects cannot be accounted for in trials using single subjects. Our response variable ("pollen" delivery) is completely plant-focused. It does not record specific bee behaviors and therefore does not address whether individual bees develop preferences, but it does efficiently capture the cumulative consequences of those behaviors for pollination service by a large group of bees foraging together in a naturalistic way.

Materials and methods

We ran experiments from October 2013 to February 2014 in indoor flight cages with supplemental fluorescent lighting. Commercial colonies of *B. impatiens* (Apidae) bumblebees, supplied by Biobest (Leamington, Ontario), were fed ad lib with pollen delivered directly to the nests. Worker bees collected sucrose solution from artificial flowers during alternate training periods and experimental periods. All experiments ran for 8 h, from 13:00 to 21:00.

The flowers use the nectar supply system described by Thomson et al. (2012), in which a capillary wick of sewing thread conveys nectar from a glass jar reservoir to a recessed nectary in the plastic lid of the jar. They dispense and capture the same Sensient® powdered food dyes as pollen analogs, but unlike the separate male and female flowers used in 2011, our new flowers are cosexual. The nectary is covered by a rigid plastic superstructure that functions as the corolla (Fig. 1). To reach the nectary, bees have to push into an opening, much as a car drives into a garage. In doing so, the bee's dorsal surface brushes against a flexible piece of sticky tape that hangs down over the opening like a half closed garage door. The tape's adhesive side faces outward, providing a "stigma" that captures some of the dye on the bee. Once inside, the bee then brushes against a narrow slot in the downward slanting ceiling, causing a dose of powdered dye to fall through

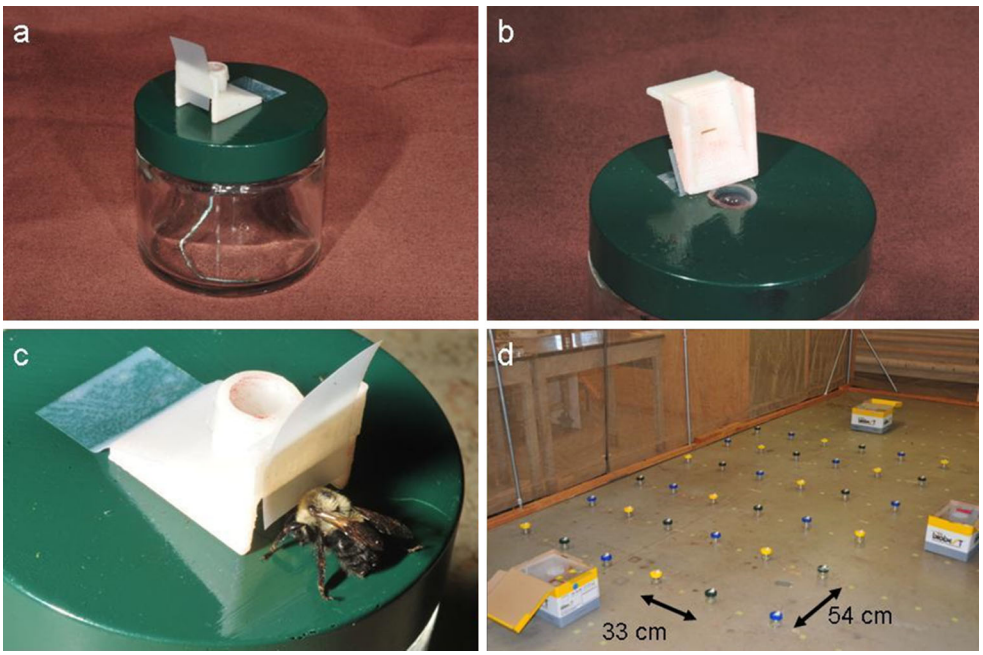

Fig. 1 Artificial flowers that simulate pollen dispersal by donating and receiving powdered food dyes. **a** Entire assembly showing the sewing thread wick that conveys nectar by capillary action from the glass jar reservoir to the nectar receptacle in the jar lid. The white plastic superstructure, made by 3D printing, is attached to the lid by tape that serves as a hinge. The upward projecting cylinder is the dye hopper. **b** The superstructure folded back on its hinge, showing the slit through which dye is dispensed from the storage hopper onto a bee. **c** A bumblebee (*Bombus impatiens*) brushing against the outward facing, sticky tape stigma as it enters a flower. **d** Interior of large flight cage, showing three commercial colonies of bumblebees and the spatial layout of Experiment 3, in which three caffeine concentrations are offered by *yellow*, *green*, and *blue* flowers, $N = 12$ of each. During actual trials, the bee colonies were located farther from the arrays of flowers

a 1-mm slit from an overhead storage hopper onto the bee's dorsum, thereby functioning as an "anther." The superstructure is held in place over the nectary by tape that acts as a hinge. The ceiling is low enough that small bees must brush against the ceiling, and the hinge allows large bees to make similar contact as they push the assembly upward. These flowers mimic the pollen and nectar presentation characteristics of species like *Penstemon* in that they replenish nectar quickly after a visit and deposit small doses of pollen on many visitors (Castellanos et al. 2006). To refine the design and dimensions of these new flowers, we used 3D printing to make variants for testing, and we used the same method to mass-produce the final version.

Initial training

We first exposed each new colony to a 5-day training phase during which they learned to seek nectar from flowers with black lids. To provide abundant nectar with no replenishment delay, we replaced the thin capillary thread with a cotton dental roll, about 1 cm in diameter, as a wick. The nectar reservoirs were filled with BIOGLUC®, a proprietary solution of sugar, preservative, and coloring agent shipped with Biobest colonies.

For the first two days of training, we exposed the bees to 3D-printed flowers that lacked dye and stigmas, so that bees could learn the location of the nectar and associate the artificial flowers with nectar rewards. We initially omitted the stigmas to provide unhampered access and thereby hasten discovery of the nectar site. We introduced stigmas for the final three days of training, so that bees became familiar with pushing in underneath the stigma to reach the nectar.

Caffeine levels

For all experiments, BIOGLUC was replaced by 30 % solutions of table sugar, either without caffeine or with 10–5 M caffeine ("low caffeine") or 10–4 M caffeine ("high caffeine"). The first two trials were pilot experiments designed to establish informative caffeine concentrations that were also biologically realistic, based on results and data from the plant genera *Coffea* and *Citrus* presented by Wright et al. (2013). Experiment 1 comprised five 8-h trials comparing decaf to high-caf, all with the same colony of bees; Experiment 2 comprised five 8-h trials comparing decaf to low-caf, all using a second colony of bees. In each of these experiments, we set out 18 flowers

of each caffeine treatment, alternating checkerboard fashion in a 6 × 6 array, with the flowers spaced 54 cm between columns and 33 cm between rows (see Fig. 1d for the general setup). Each trial began with clean flowers, freshly loaded with Sensient® FD&C Red 40 dye, and equipped with fresh stigmas. At the end of a trial, we harvested each stigma, dissolved the accumulated dye in 5 ml of water, and determined the amount of dye with a spectrophotometer (details in Thomson et al. 2012 and Cembrowski et al. 2013). The caffeine treatments were indicated by jar lids that had been spray painted with Krylon Fusion® paint for plastics in Patriotic Blue #42329 and Sunbeam (yellow) #42330 colors. We reversed the color- caffeine associations after each trial to avoid confounding color effects and caffeine effects. After each trial, we exchanged the flower colors and set up the next experiment. However, before recording any responses, we let the bees forage on the new flowers (without dye or stigmas) for a relearning period of 36 h. After that period, we added dye to the hoppers and installed fresh stigmas. Caffeine level was signaled only by the color of the jar lids; all of the 3D-printed superstructures were unpainted whitish plastic.

After the pilot trials confirmed that our caffeine concentrations did affect dye delivery, we set up the definitive Experiment 3, which presented all three caffeine levels simultaneously for six 8-h trials. We added a third color of jar lids (Krylon Hunter Green #42324) so each caffeine level would again be paired with a distinctive color, and our trials rotated through all six possible combinations of caffeine level and color. Each trial comprised 12 flowers of each type, interspersed in a 6 × 6 array as shown in Fig. 1d. As before, we allowed the bees to relearn color caffeine associations for 36 h between trials. To reduce the possibility of getting unrepresentative foraging by a bee colony with idiosyncratic behavior, we used three new colonies of bees, all freely foraging at the same time. We did not attempt to count the number of visits, but we can make a minimum estimate as follows: casual observations found approximately three bees foraging at a time. Conservatively, adopting a foraging rate of five flowers per minute yields an estimate of 2,400 flower visits per 8-h trial or a total of 16,400 visits for the set of six trials. This number is extremely imprecise but is probably an underestimate.

Statistical analysis

We treated the quantity of dye received by each flower as an independent response measure. Such treatment is warranted by the complete spatial interspersion of the different flower types (Hurlbert 1984). We converted each flower's mass of dye received to a fraction by dividing it by the total mass received by all flowers in that day's trial. This conversion equalizes variation in the total amounts of dye that might be transferred on different days, because flower loading is imprecise and because the activity of the worker force of bees waxes and wanes as colonies develop. It also allows us to pool data from all trials within an experiment. Because the data were well-conditioned, we applied simple, fully balanced, two-way factorial ANOVA (computed with the *anova* package of R version 3.0.0 (R Core Team 2013) to the pooled data from the several trials within each of the three experiments. The response variable was the fraction of dye found on a flower, and the treatments were color (blue or yellow in Experiments 1 and 2; blue, yellow, or green in Experiment 3) and nectar type (zero, low, or high caffeine).

Results

Dye hoppers still contained residual dye at the end of a trial, in accordance with our testing that showed this flower design to dispense small amounts of dye per visit. Some bees actually built up visible pellets of dye on their hind tibiae, indicating that they had groomed dye from their body hairs and packed it in their corbiculae, much as they would do with real pollen (Figs. 2, 3).

Flower color did not affect dye receipt in any of the three experiments (all P values for flower color effects were greater than 0.27), and there were no significant interactions between flower color treatment and caffeine treatment. Therefore, we report caffeine effects only. Caffeine effects were significant in all experiments, but depended on dosage. In Experiment 1, the 10^{-4} M caffeine flowers received significantly less dye than did caffeine-free flowers (46.7 % of the total dye transferred in all trials; $F_{1,173} = 4.14$, $P = 0.044$), so caffeine at this concentration reduced "pollination" service, probably through a mild aversive effect on bees. The effect of caffeine was reversed at the lower caffeine concentration in Experiment 2: the

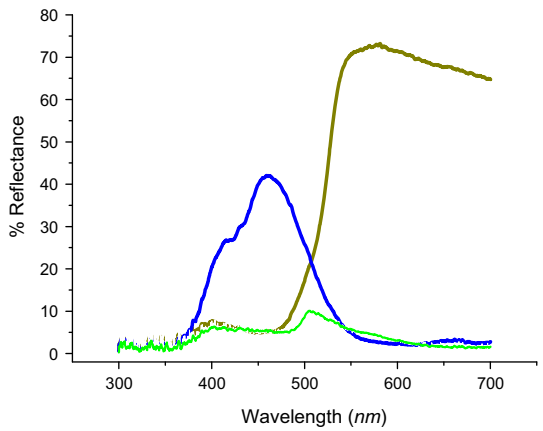

Fig. 2 Reflectance spectra of the three paint colors used

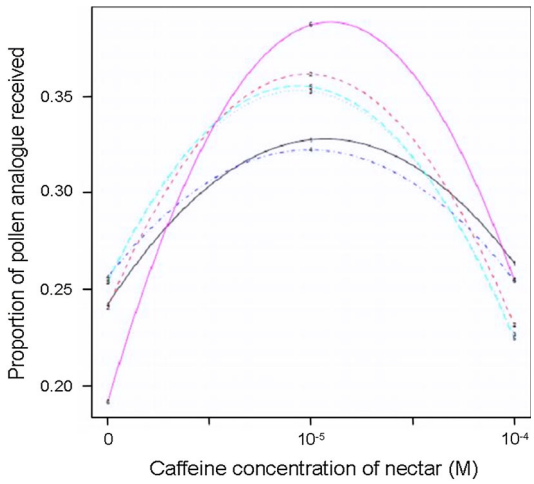

Fig. 3 Dye receipt by each of the three treatment levels in six trials of Experiment 3. *Points* indicate the amount of dye delivered to all 12 stigmas of a given caffeine level, expressed as a fraction of the total amount of dye delivered in that trial. To show which points come from which trial, points within a trial are connected by lines representing quadratic fits

10^{-5} M caffeine flowers received 53.0 % of all the dye transferred ($F_{1,173} = 6.88$, $P = 0.001$), consistent with a mild preference.

The results of Experiment 3 were consistent with the pilot Experiments 1 and 2. For the whole data set, flower color effects were insignificant ($F_{2,202} = 0.88$, $P = 0.41$), as was the interaction of flower color with caffeine level ($F_{4,202} = 0.55$, $P = 0.69$), but the caffeine effect was highly significant ($F_{2,202} = 28.1$, $P = 1.7 \times 10^{-11}$). On average, the low-caffeine (10^{-5} M) flowers received 42.1 % of the dye (range across the 6 trials 38.7–46.5 %). Controls with no caffeine received 28.8 % (range 23.0–30.8 %), and the high-caffeine flowers received 29.1 % (range 26.9–31.6 %). We conducted pairwise comparisons of caffeine treatments by dropping one level at a time and rerunning the analysis. Low caffeine differed significantly from the other two treatments ($P < 10^{-7}$ for both), but high caffeine and no caffeine did not differ ($P = 0.83$). The greater dye receipt of the low-caffeine flowers was consistent across all six trials of Experiment 3. We regard Experiment 3 as more conclusive than the first two, because the use of multiple bee colonies reduces the possibility of spurious results from an aberrant colony.

Discussion

Bee preferences and pollination success

Our results confirm (for bumble bees) the speculations by Singaravelan et al. (2005) and Wright et al. (2013) that low concentrations of caffeine in nectar could confer improved pollination success on flowers, despite the aversive effects of this potent alkaloid at higher concentrations. A moderate concentration of caffeine in nectar consistently optimized pollen receipt in our experiments, where flowers offered clear color cues indicating the type of nectar they offered. We believe that this effect probably arose because bees generally prefer this type of nectar, perform rapid associative learning of the matching color, and switch fluidly to different colors as circumstances changed. Such learning would not be surprising, for bees are well-known to form and reform associations rapidly (Dukas 1995, Menzel and Müller 1996). However, our experimental apparatus is focused on the consequences for the plants rather than the behavior of the animals. Because we did not track the numbers of bee visits, we cannot rule out the possibility that bees visited all of the flowers types equally frequently, but simply transferred more dye particles per visit to the low-caffeine types. Such an effect could arise, for example, if they stayed longer or moved around more on the low-caffeine flowers (see Thomson et al. 2012). Regardless of the behavioral mechanism, the results surely arise from the way that low-dose caffeine affects bumblebees' interaction with the flowers.

Although our experiments do not distinguish whether the effects operate through visit number, visit quality, or a combination of the two, we view this as a consequence of modeling realistic conditions. If we wish to judge the effects of nectar additives on plant pollination under natural situations, we need to allow both classes of effect to occur simultaneously. In the real world, pollination service is provided by many bees from many colonies, all of them making decisions from individual and social information (Kawaguchi et al. 2007; Avarguès-Weber and Chittka 2014) and all of them influenced by each other's choices through the continuing depletion of nectar. Experiments in which plant reproductive success is estimated purely from visitation preferences are likely to miss relevant components of behavior, especially if individuals are allowed to forage singly. If numerous individuals are allowed to forage simultaneously, as in our experiments, keeping track of individuals becomes burdensome.

Caveats: dye transfer characteristics and male reproductive success

By using a single dye color, we are effectively measuring only the female component of pollination success. It is conceivable, although unlikely, that the advantage shown by low-caffeine flowers in dye *receipt* might be offset if other nectar types were more proficient in dye *donation*, i.e., the male component of pollination success. In principle, we could test this by having the flowers of the three nectar types dispense three different colors of dye. The

spectrograms of stigma loads could then be decomposed algebraically to determine the amount of each color received. We chose not to add this level of complexity to the caffeine experiment because (1) in analogous trials using two colors of dye and two concentrations of sucrose, male and female success strongly covaried (Tan and Thomson, unpublished), and (2) we have not be able to envision a plausible mechanism by which donation and receipt might differ in a design as simple as these caffeine trials.

Because our present experiments use a single dye color, it is not possible to distinguish "self" from "outcross" deposition. In a real plant, it would be valuable to make this distinction in evaluating the relation between pollination and realized reproductive success. Despite this, total stigmatic loads of pollen are often used as estimates of "female success" in real plants, and we adopt that convention here. Our claim that moderately caffeinated nectar results in more pollination should translate robustly to real plants unless there is some strong interaction between caffeine level and selfing rate. We do not see a likely mechanism for such an interaction. In any case, our flowers have low selfing rates: in a test array of identical flowers that offered one of two different dye colors, flowers received only about a 3.5 % excess of their own dye color.

We have not examined how closely the transfer of powdered food dyes matches the transfer of pollen by bumblebees in any real flower, but this has been done for powdered fluorescent pigments and pollen of the lily *Erythronium grandiflorum* (Thomson et al. 1986). Those authors concluded that dye particles moved farther and were deposited on more stigmas than pollen grains, but that the quantities deposited on stigmas were sufficiently correlated for dye deposition to reliably predict pollen deposition. That conclusion probably holds for food dyes, also, but direct trials would be warranted.

Our experiments included color cues that signaled the caffeine status of flowers, making it easy for bees to discriminate. Therefore, these experiments are not directly aimed at the basic evolutionary question of whether a mutant plant that produced caffeinated nectar could invade and spread in a population of non-caffeinated plants that are otherwise identical. That question would hinge in part on system-specific salience characteristics that would allow pollinators to remember the locations of preferred plants, such as plant size, plant spacing, numbers of flowers, and availability of landmarks. Nevertheless, bumblebees in particular have been shown to locate plants with more nectar and visit them more frequently in manipulative experiments in both the field (Thomson 1988; Cartar 2004) and lab (Makino and Sakai 2007), so analogous discrimination based on nectar chemistry is at least plausible.

In practice, the evolution of secondary metabolite levels in nectar would probably be constrained by the tendency for nectar concentrations to be correlated with foliar concentrations (Manson et al. 2013a), and foliar concentrations are likely to be under selection through folivores. Therefore, selection for optimal concentrations of compounds like alkaloids in nectar would probably take the form of regulating the amount of "leakage" from other plant parts. We would predict that attractive or addictive compounds would have a higher ratio of foliar concentration to nectar concentration than would purely aversive compounds.

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