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— Note on Methodology —

ESTIMATING POLLINATION SUCCESS WITH NOVEL ARTIFICIAL FLOWERS: EFFECTS OF NECTAR CONCENTRATION

James D. Thomson[1,2]*, Jane E. Ogilvie[1,2], Takashi T. Makino[1], Angela Arisz[1,3], Sneha Raju[1,3], Vanessa Rojas-Luengas[1,3], and Marcus Guo Rui Tan[1,3]

[1]*Department of Ecology and Evolutionary Biology, University of Toronto, 25 Harbord Street, Toronto, ON, M5S 3G5, Canada*
[2]*Rocky Mountain Biological Laboratory, Post Office Box 519, Crested Butte, CO, 81224-0519, USA*
[3]*Undergraduate contributors listed alphabetically*

Abstract—We developed novel artificial flowers that dispense and receive powdered food dyes as pollen analogues while their nectar is replenished by capillary action. Dye receipt, which can be measured colourimetrically, is a direct surrogate for pollen receipt or female reproductive success, but can also serve to compare pollen donation (male reproductive success) from flowers with different colours of dye. By allowing captive bumble bee colonies to visit large arrays of such flowers, we investigated whether total dye receipt depended on the sugar concentration of a flower's nectar. Estimating pollen transfer, rather than simply visitation rate, is appropriate for this question because flowers with more concentrated nectar might accrue more pollen not only through higher visitation rates but also through longer visits that transfer more pollen per visit. Flowers with richer nectar did receive more dye regardless of their spatial arrangement, but the effect was greatest when rich and poor flowers were segregated in large blocks, as opposed to being intermingled.

Keywords: bumble bee, food dye, male fitness, pollen analogue, pollinator visitation, reproductive success

INTRODUCTION

Experiments involving real pollinators visiting artificial flowers, or altered real flowers, have a long history in the investigation of plant-pollinator relationships. Clements & Long (1923) summarize several decades of early experiments by Plateau and many others, all apparently stimulated by Vallete's controversial report of a *Macroglossa* hawkmoth trying to feed at a floral-patterned tapestry, "passing from one bouquet to another and choosing skilfully the flowers that it sought to probe" (Clements & Long 1923, p. 136). The principal questions at this time concerned the characteristics of flowers that induce insects to visit, and Clements and Long (p. 3) credit Plateau with introducing the experimental method to plant-pollinator studies. More recently, experimenters have used artificial flowers to examine general behavioural tendencies that might characterize pollinator foraging with consequences for plant reproductive success, such as movement rules (Waddington & Heinrich 1979), flower constancy (Gegear & Laverty 2005), nectar preferences (Cnaani et al. 2006), risk avoidance (Real 1981), frequency-dependence (Smithson & Macnair 1997), traplining (Ohashi et al. 2008), and modes of learning (Chittka & Thomson 1997, Biernaskie et al. 2009). Much of this work has used bumble bees because of their amenability to experimental situations and their recent commercial availability.

Artificial flowers have therefore gained a secure place in the investigation of pollination, but we argue that this tool has not been fully exploited. Most often, individual bees have been observed while they work alone. Flowers are typically loaded with honey water or sugar solutions; replenishing these rewards during trials is a logistical challenge that can add expense and limit experimental designs. More fundamentally, the response variables are almost always the numbers, durations, or patterns of visits, and the *direct* questions concern pollinator behaviour. To the extent that the results are *indirectly* interpreted in terms of fitness consequences to plants, those consequences are usually extrapolated from visitation data, on the qualitatively reasonable but quantitatively weak assumption that fitness increases with visitation. One exception is the work by Stone & Thomson (1994), in which artificial flowers were furnished with "anthers" that presented real pollen grains for transfer by bumble bees to artificial "stigmas" that retained grains for counting. This arrangement provided response variables based directly on pollen delivery. It was used to test hypotheses for the evolution of heterostyly (Lloyd & Webb 1992) that depended on the relative pollen-donation proficiency of different style-length morphs.

Here, we extend Stone and Thomson's approach, describing a new design of "pollen-donating-and-receiving" flowers designed for general experiments with bumble bees in large flight cages. Envisioning more naturalistic experiments

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*Corresponding author; email: james.thomson@utoronto.ca; telephone: 1 416 927 0493; fax: 1 416 978 8532

that involved exposing large arrays of flowers to *ad lib* foraging by entire colonies of bees for hours to days, we needed low-maintenance flowers that were inexpensive, simple, sturdy, and easy to score. The novel aspect of the flowers described here is the use of powdered food dyes as pollen analogues, so total pollen transfer over a long period of visitation could be measured by dissolving "stigma" loads in water and measuring the amount of dye by spectroscopy. The criteria of simplicity and low maintenance were met by adapting Makino & Sakai's (2007) successful design for continuously conveying nectar from a reservoir to a flower by capillary action. We have typically set up an experiment during an evening after the bees have returned to their nest, allowed the bees to forage unattended on the following day, and then harvested the experiment on the second evening. (This schedule is particularly convenient for student investigators with daytime classes.)

To demonstrate a simple application, we here extend the demonstration by Cnaani et al. (2006) that *Bombus impatiens* workers strongly prefer nectar rewards of high concentration over those of low concentration, even when net caloric returns are similar. We asked whether female flowers offering 30% sucrose would receive more pollen delivery than visually identical flowers offering 10%, and whether that advantage depended on spatial arrangements of flowers that either impeded the task of learning where the richer flowers were (completely intermingled phenotypes) or eased it (aggregated blocks of phenotypes). In addition to testing for nectar-concentration treatment effects on the dye received by a flower, we also examined incidental spatial effects: distance from the nestbox, and edge versus centre of array.

MATERIALS AND METHODS

Flower design and function

Our current flower design (Fig. 1) comprises a widemouth glass screwtop jar (60 mL, #89043-374; VWR International, Mississauga, Ontario, Canada) with a 9 mm hole drilled in the centre of the 55 mm lid to receive a nectar cup. A 5 mm thick ring, sliced from nominal 1/2 –inch PVC electrical conduit (actual internal diameter = 15.5 mm), is attached to a sanded lid with epoxy cement, creating a receptacle around the centre hole for anthers or stigmas. This assembly is painted with "Patriot Blue" Krylon Fusion spray paint for plastics. The nectar cup is a conical-bottomed polyethylene tube sold for embedding tissue specimens for microscopy (BEEM embedding capsules, #130-SPC; Ted Pella Inc., Redding, California, USA). We shorten these tubes by removing 7 mm from the open end (length of the nectar cup = 12 mm), and then use an embroidery needle to puncture the bottom of the cone so that a length of fine polyester-cotton sewing thread can pass through. Following Makino & Sakai (2007, Appendix S1), we create a nectary by tying together two pieces of thread. The upper end of the nectary is made up of a thicker thread that is looped and knotted around a thin piece of wire to create two small loops. The lower thinner thread extends into the nectar reservoir and when dipped into sugar solution, the upper loops accumulate nectar; bees can extract sugar solution by licking with the glossa. We tested flowers that lacked nectar cups, but found that the nectary knots tended to dry out if exposed at the top of the jar lid. Also, when flowers lacked nectar cups, many bees could forage simultaneously on a

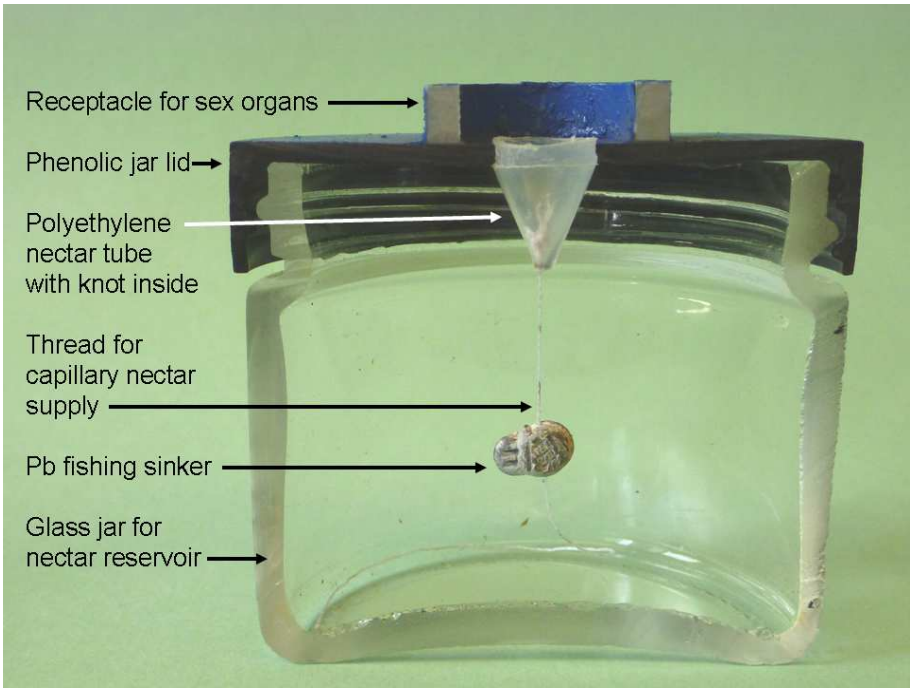

FIGURE 1. Cross-section of the basic dye-donating capillary artificial flower, showing the blue-painted plastic jar lid on top of the glass jar that serves as a nectar reservoir. Note the raised ring-shaped receptacle on the jar lid that receives the anther or stigma (anther and stigma not shown), the conical nectar cup, and the capillary thread that hangs from the nectar cup down into the nectar reservoir, weighted by a roughly spherical "split-shot" fishing sinker made of lead.

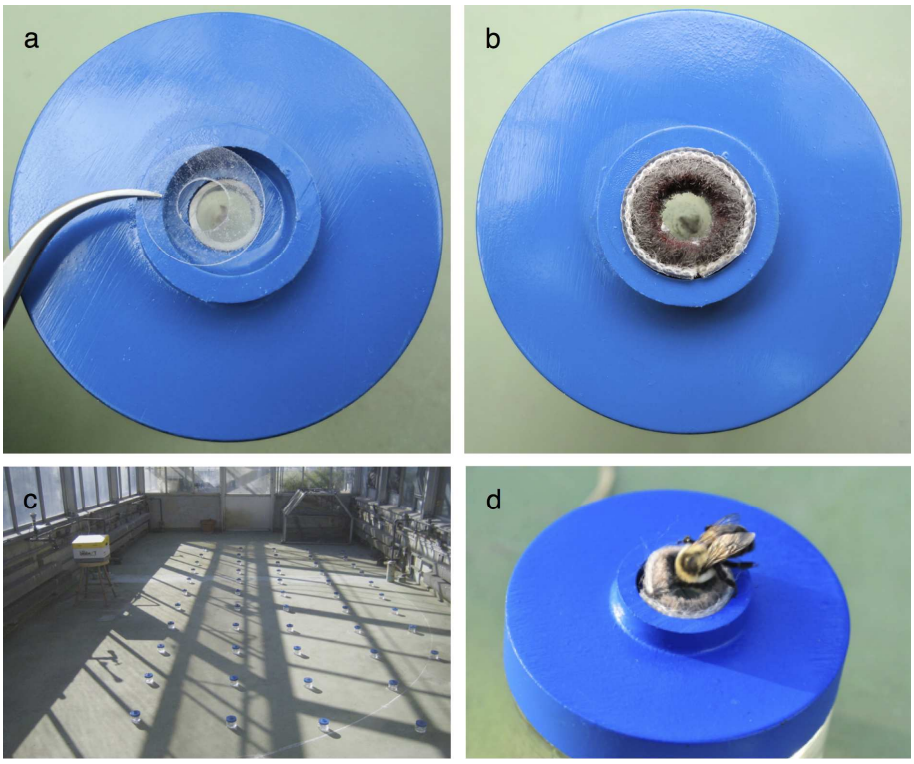

FIGURE 2. Photographs of artificial flowers (a) with an adhesive stigma being placed inside the receptacle with a pair of forceps, (b) with a fibrous anther, (c) positioned in a typical array in the greenhouse with the box holding a commercial colony of *Bombus impatiens*, and (d) with a nectar-feeding bumble bee worker.

flower; adding the nectar cups restricted flower access to one bee at a time, which is a characteristic that we preferred. Because bees sometimes pull on the thread after grasping the knot with their mandibles, we secure the nectary knot by adding a split-shot fishing sinker to the hanging end of the thread.

Our artificial flowers are female or male, representing diclinous or dichogamous plants. A "female" flower receives a "stigma" made from a transparent, self-adhesive reinforcement made for strengthening holes punched in paper (Staples Portable Reinforcements; Staples Canada, Richmond Hill, Ontario, Canada; Fig. 2a). We spray a sheet of these reinforcements with a light coat of adhesive (3M Spray Mount Artist's Adhesive; 3M Canada, London, Ontario, Canada), remove them from the backing sheet with fine forceps, and place them in the receptacle, sprayed-side-down. Pressing the stigma into the receptacle with stabbing motions of the forceps points adheres it sufficiently so that it will not be dislodged by bees reaching through it to feed. The factory-coated upper side provides a uniform adhesive surface to which dye particles can adhere when nectar-seeking bees brush against the edge of the nectar cup. In contrast, a "male" flower receives an anther made from stiff, pile weather-stripping material made for sealing metal windows (Tago pile replacement #74026, Fig. 2b). We cut strips to a constant length that can be curled into circles that fit precisely into the receptacles, with the fibres facing inward. At the beginning of an experiment, we load each male flower with Sensient powdered food dye (Sensient Colours Canada, Kingston, Ontario, Canada) in the following manner designed to create approximately similar anther loads: we dip a Microbrush applicator (regular size, #1000; Microbrush, Grafton, Wisconsin, USA) in a container of dye powder, remove excess by tapping the applicator in a stereotyped way, and apply powder to the anther fibres by rolling the applicator around approximately half of the circular anther while pressing the applicator against the anther fibre. We repeat the process for the other half of the anther. The mechanical and electrostatic properties of these materials are such that a flower loaded in this way can dispense dye for tens to hundreds of visits. Bees that regularly visit male flowers develop visible deposits of dye powder on the hind legs. Each flower in an experiment receives a unique identity flag made from tape.

Scoring dye transfer

At the end of an experiment, we use clean forceps to lift out each stigma and transfer it to a clean test tube containing 8 mL of water delivered from a bottle equipped with a repeating dispenser nozzle (Biohit Prospenser, 10 mL size; Biohit, Helsinki, Finland). We also transfer the identifying flag to the tube. To promote complete solution of dye adhering to the stigma, we agitate the tube for 8 s on a vortexer. Dye concentration can be most accurately assessed with a spectrophotometer, but for the pilot experiments described here, we simply ranked the tubes visually from most to least intense colour. A long glass-sided rack made it convenient to arrange the tubes in order. For analyses, the response variable was the dye-intensity rank. Although rank data are frequently associated with non-parametric tests used when data fail to meet the assumptions of parametric analysis, ranks are completely amenable to parametric tests when they meet those assumptions (see Conover and Iman 1981), so for simplicity we tested for significance of treatment effects by *t*-tests on rank scores or two-way factorial ANOVAs when testing for effects of secondary

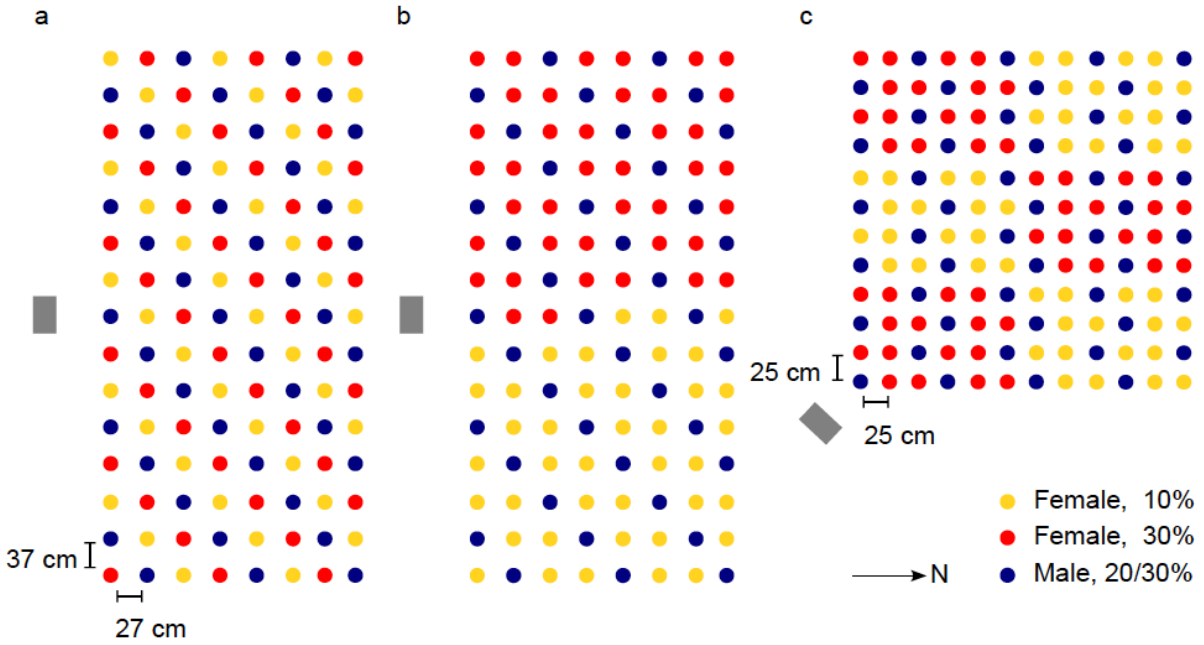

FIGURE 3. Designs of the artificial flower arrays for Experiments 1-3. All flowers were painted blue; colours in this figure simply denote different flower types. In Experiment 1 (a), rich (30%-sucrose nectar) and poor (10%-sucrose nectar) female flowers were intermingled throughout the array. In Experiment 2 (b), the two female flower types were spatially clustered into two large patches comprising the western and eastern halves of the array. Experiment 3 (c) imposed an intermediate degree of clustering. Experiments 1 and 2 were conducted in a greenhouse and had equivalent flower spacing, while Experiment 3 was conducted in an indoor flight cage with closer flower spacing. In Experiment 1, males had 20%-sucrose nectar, while in Experiments 2 and 3 the males had 30%-sucrose nectar. Grey rectangles show the position of the *Bombus impatiens* colony box for each experiment.

positional factors, as noted. For testing effects of nectar concentration of dye receipt, the results of Thomson (1986) and Cnaani et al. (2006) justify one-tailed tests because there is a clear *a priori* expectation that dye receipt should increase with nectar concentration. We used two-tailed probabilities for testing positional effects because we lacked *a priori* expectations. For an index of the strength of treatment effects, we divided the absolute difference between the mean ranks of the treatments by the number of flowers per treatment. This index, which requires that treatments have equal numbers of flowers, ranges from 0 (no effect) to 1 (no overlap in ranks).

Experiments

Our pilot experiments were conducted in a greenhouse bay (Fig. 2c). The arrays comprised equal numbers of three flower types: male flowers (loaded with FD&C Red 40 dye powder), "rich" female flowers offering a 30% solution (weight/volume) of table sugar, and "poor" female flowers offering a 10% solution.

Each experiment used a commercial colony of *Bombus impatiens* bumble bees (Biobest, Leamington, Ontario, Canada). We removed the factory-supplied nectar reservoir, placed the nestbox at one side of the array, and opened it to allow the bees free access to the flowers (see Fig. 2d for a photograph of a bee visiting an artificial flower). We supplied pollen *ad lib*, directly to the comb. To train the bees before conducting experiments, we supplied a mixture of male and female flowers with 30% sucrose. To speed the discovery and learning phases, and to prevent the nectaries from drying out, we added a drop of 50:50 honey water to the nectar cup of each training flower. After a few individuals discovered the flowers and began foraging, the behaviour spread quickly. No honey was used after this initial effort to induce visitation. We typically had active foraging forces of 5-15 workers for experiments, but we did not try to quantify this dynamic variable.

After the worker force was trained for 2-3 days, we began experiments. We replaced arrays of training flowers with experimental arrays after dusk, when the bees had returned to the nest. Each experiment ran from dawn to dusk on the second day, with the bees free to forage. In Experiment 1, we stocked the male flowers with 20% sucrose. We observed, however, that most bees began avoiding the males. We suspect that bees tend to avoid the male flowers because the fibrous anther hinders access to the nectary. We feared that dye transfer might be negligible, but in fact we were able to rank the tubes without difficulty. Before continuing with other experiments, we re-trained the bees by exposing them to a training array with male flowers only, with 30% sucrose. In the other experiments, we stocked males with 30% solution, which sufficed to keep bees visiting them.

We conducted three successive experiments in which rich and poor female flowers were presented in different clustering patterns (Fig. 3): 80 fully intermingled (or no clustering); coarse clustering (two patches of 40 each); and intermediate clustering (six patches of 16 flowers each). In

all cases, male flowers were interspersed evenly through the array. In experiments 1 and 2, flowers were positioned in 8 by 15 arrays, with intervals of 27 cm between columns of flowers and intervals of 37 cm between rows of flowers (Fig. 3a and b). The third experiment represents different experimental conditions: after discovering undesirable spatial effects in the greenhouse (below) we moved to a smaller cage in the lab with more uniform lighting. The cage dimensions imposed closer flower spacing and square arrays; male and female flowers were presented in a 12 by 12 array at intervals of 25 cm (Fig. 3c). Each of the three spatial designs was run once, for a single foraging day. We used a single colony of bees for all three experiments.

RESULTS

In Experiment 1 (no clustering), female flowers with 30%-sucrose nectar received significantly more dye than those with 10%-sucrose (index = 0.25; *t*-test on rank scores, t_{78} =1.95, one-tailed P = 0.027). We explored additional spatial effects with two-way analyses of variance, including nectar concentration. Proximity to the nest did not affect dye receipt, nor did position within the array (edge versus central flowers), nor did these factors interact significantly with nectar concentration ($P > 0.2$ for all tests). There was, however, a significant bias toward higher receipt in the western half of the array than in the eastern half (*t*-test, t_{77} = 3.06, two-tailed P = 0.003). Because the 30% and 10% flowers were spread evenly across the array, this bias does not affect the nectar treatment effect. Either the bees conferred more visits to the 30% flowers or those visits transferred more pollen analogue, or both. Casual observations (not timed by watch) suggested that visits to 30% flowers typically lasted 5-6 s, whereas visits to 10% flowers lasted only 1-2 s, making it reasonable to suppose that more dye might be transferred per visit. Because of the intermingling of the rich and poor female flowers, it would be difficult for bees to remember their locations and visit them selectively.

Experiment 2 (coarse clustering) was conducted immediately following Experiment 1, so the same conditions applied, and most of the bees would have been the same. The nectar treatment effect was very much stronger, with the 30% flowers receiving decisively more dye (index = 0.76; *t*-test on rank scores, t_{78} = 7.68, one-tailed $P = 1.52 \times 10^{-11}$). Unfortunately for a clear interpretation of this result, the 30% flowers were all located in the western half of the array, which Experiment 1 had shown to be more favoured, irrespective of nectar concentrations. The strong dye-donation advantage of rich flowers shown in Experiment 2 therefore probably includes three components: more dye donation per visit, attributable to the nectar treatment; higher visitation to flowers in the large rich cluster, also attributable to the nectar treatment; and an additional component of visitation attributable to the bees' unexplained preference for the western end of the array.

The intermediate clustering in Experiment 3 resulted in an intermediate treatment effect (index = 0.29; *t*-test on rank scores, t_{94} = 2.50, one-tailed P = 0.007), still significant.

DISCUSSION

Effects of nectar concentration

Although these pilot experiments were intended as demonstrations of the utility of the new dye-donating capillary flowers, rather than as a comprehensive research program into nectar-concentration effects, they sufficed to confirm that flowers with richer nectar receive more dye than those with dilute nectar. To the extent that these dyes serve as accurate pollen analogues, these results confirm the reasonable expectation that the strong preference of *Bombus impatiens* workers for concentrated nectar shown by Cnaani et al. (2006) is likely to translate into higher pollen receipt for flowers with richer nectar. Thomson et al. (1986) discuss the adequacy of other (fluorescent) powdered dyes as pollen analogues, concluding that there are some significant differences in transport but that more dye transport will generally indicate more pollen transport. We expect that this same rough concordance will apply to the Sensient food dyes we used here.

The different strengths of nectar-treatment effects in the three experiments also indicate that the effects of floral phenotypic variation on pollinator behaviours—and therefore, plant reproductive success—will be sensitive to the spatial arrangement of those phenotypes. We expect that the treatment effect was smallest when the two nectar treatments were completely intermingled (Experiment 1) because the bees had difficulty remembering the locations of the richer flowers. If they completely lacked the cognitive ability to confer higher visitation rates to the richer flowers, the entire treatment effect would presumably be attributable to differences in the quality of visits. The most important such difference is probably the longer duration of visits to richer flowers, which can result in more dye transfer, especially if the bees shift their positions during visits (see Thomson 1986 for analogous experiments with real flowers). As floral phenotypes become aggregated into larger clusters, it becomes easier for bees to concentrate their visits on the rich clusters. This can occur through at least two mechanisms: first, bees can learn the locations of the more rewarding clusters (see Cartar 2004, Makino & Sakai 2007); second, appropriate movement rules can produce area-restricted search patterns of flight distances and turning angles that tend to keep bees in richer clusters (Pyke 1978). A strength of our new method is that it integrates the effects of all such mechanisms in a way that plausibly measures overall "female fitness."

The next step is to extend such investigations to estimate "male fitness" by measuring dye export from male flowers with rich and poor nectar phenotypes. Such experiments are possible by employing different colours of dye on the two male phenotypes. The resulting solutions of stigma loads will contain mixtures of both colours, but the relative concentrations of the two colours can be estimated by mathematically decomposing the overall absorbance spectrum into the two components of the mixture (a least-squares procedure is detailed by Harris (2007, pp. 402-46)).

Considerations on the use of dye-donating capillary flowers

The pilot experiments confirm three advantages of this design over other approaches: simplicity of construction and nectar replenishment, unattended operation, and a closer approximation of the pollination process, including the ever-elusive "male success." By measuring dye transfer rather than visitation rate, this design transfers the focus of investigation from the pollinator to the plant, and bypasses the necessity of inferring plant reproductive success from visitation rates. We can say that most real flowers do present pollen somewhat gradually through time (see Harder & Thomson 1989); they also replenish nectar after draining, at least to some extent (Willmer 2011); and they typically accrue pollination success incrementally through the actions of numerous visitors. Those visitors typically have access to many flowers, and can learn about particular floral characteristics and spatial locations over time. With numerous capillary flowers deployed in large flight cages, all of these elements can be simulated.

The artificial flower design has important limitations, however. First, although we can quantify the amount of dye reaching the stigmas of female flowers, we have no good way of quantifying the amount of dye made available in the male anthers, nor do we have a clear picture of how long the dye remains available for dispersal as visits accumulate. Similarly, although Makino & Sakai (2007) give some rates of nectar replenishment for such flowers, those measurements are approximate. We do not know how nectar refilling rates vary with the type or the number of capillary threads, with the viscosity of the nectar, or with physical factors such as temperature and humidity. Second, the correspondence of dye and pollen transport characteristics remains uncertain.

We can envision further studies or methodological variations that address some of these issues. To quantify dye application, it might be possible to apply concentrated solutions of dyes to fibrous anthers by microcapillary tubes of known volume, then let the fibres dry before presenting the flowers. If the dried dye can be flaked off by subsequent insect visits, this liquid application might provide an extended period of dye donation. To escape the discordant transport characteristics of dye and pollen, we could simply use real pollen grains (following Stone & Thomson (1994)), rather than powdered dyes, although this would require a reversion to time-consuming counts of stigma loads. To retain the advantage of spectrophotometry, it might be possible to use microencapsulation techniques to embed the powdered dye into pellets whose size and electrostatic properties more closely resemble pollen. Alternatively, a slurry of real pollen grains could be suspended in continuously stirred liquid dye while precise aliquots are withdrawn by micropipette and transferred onto anther fibres to dry. The dyeing process would presumably disrupt the pollenkitt and therefore affect the transport characteristics of the grains, but at least the dyed grains would be the right size and might retain some of their original electrostatic properties.

Although varying sugar concentration is the simplest way to manipulate floral rewards in these flowers, it should also be possible to vary the rate of capillary replenishment by adjusting the type or number of nectar-conveying threads. The storage capacity of the nectary should also be adjustable by tying smaller or bulkier knots. Flowers could also be designed to include multiple nectaries. The design is also well-suited for exploring the effects on pollinators of minor nectar constituents such as alkaloids (cf. Gegear et al. 2007).

Investigators interested in experimenting with Sensient food dyes but reluctant to purchase them in bulk should contact the senior author for samples.

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