INTRODUCTION

The evolution of biotic resources can be profoundly influenced by the behavior of animals using those resources (Mallet and Joron 1999; Bond and Kamil 2005; Grabowski and Kimbro 2005; Jalvingh et al. 2015). A classic system in which these influences have been studied is the plant–pollinator mutualism. In this mutualism, angiosperm plants offer floral “rewards” to pollinators in return for the service of pollen transfer. Much of the extraordinary diversity in floral form among angiosperm species has been ascribed to the effect of pollinator foraging preferences on floral display evolution (Schiestl and Johnson 2013). These preferences can be either innate or learned. Innate preferences can either reflect adaptation on the part of the pollinators or exploitation of a pre-existing sensory bias by plants. Preferences formed through associative learning are generally beneficial for the pollinator and are particularly interesting because they can lead to rapid diversification among plant taxa in both floral signals and floral rewards.

Although learned preferences have been shown in all kinds of pollinators from vertebrates to insects (e.g., Fukushima 1989; Lunau 1992a; Kelber and Pfaff 1997; Weiss 1997; Hurly and Healy 2002; Chittka and Thomson 2005), they have been particularly well documented in bees. In fact, the role of experience in shaping bee foraging responses has been so well studied that bees are considered a model system for the study of learning (Giurfa 2007; Menzel 2012; Leonard and Masek 2014). Bees learn an impressive assortment of floral properties, including colors, iridescence, patterns, polarization, scents; petal microtexture; and even electrical fields (Giger and Srinivasan 1995; Gumbert 2000; Whitney, Chittka, et al. 2009; Whitney, Kolle, et al. 2009; Clarke et al. 2013; Schiestl and Johnson 2013; Foster et al. 2014). Learning is associative, with responses to one of the aforementioned floral traits being enhanced when paired with a floral reward. Learned preferences in bees are commonly strong and durable (Giurfa 2007 and references within), giving them the potential to shape the evolution of floral traits.
Because bees can learn diverse stimuli, even evolutionarily novel stimuli, their learned preferences may contribute significantly to the rapid diversification of floral form that has occurred in bee-pollinated plants. Indeed, such learning is believed to have facilitated the evolution of a remarkable degree of diversity in floral display traits such as corolla shape and color, floral scent, and nectar guides (cf., Lewis 1993; cf., Chittka and Thomson 2005; Leonard and Papaj 2011; Leonard et al. 2011a; Hopkins and Rausher 2012).

A complete accounting of evidence of learning by pollinators and its impact on floral trait evolution could easily fill a full-length book (e.g., Chittka and Thomson 2005). It is thus noteworthy that virtually the entirety of research on floral learning in pollinators, including bees, concerns nectar rewards. Yet angiosperms offer pollinators a diversity of floral rewards in exchange for pollinator services, including pollen, oils, scents, nectar, and shelter (Simpson and Neff 1981; Feen et al. 2004; Seymour and Matthews 2006; Waser and Ollerton 2006; Luo et al. 2010). In particular, nectar is just one of 2 dominant rewards offered by plants, the other being pollen (Simpson and Neff 1981; Morse 1982; Kevan and Baker 1983; Kitaoka and Nich 2009). Whereas nectar is the primary source of carbohydrates for most pollinators, pollen is the principal source of protein and a critical component of the diet of developing larvae for bees and many other insects (cf. Kevan and Baker 1983; Nicolson and van Wyk 2011). All angiosperm species produce pollen, the male gamete in sexual reproduction, and in many, pollen is extracted by foraging bees, often in addition to nectar. Significantly, at least 6% of angiosperms (around 22,000 species) offer only pollen rewards in exchange for the service of pollination (Vogel 1978; Buchmann 1983; Buchmann SL, personal communication).

Pollen-only species show patterns of floral morphology that suggest pollinator behavior has shaped floral evolution. For example, pollen-only species in many different plant families show a pattern of floral morphology termed the solanoid flower form (Faegri 1986; Figure 1). Have pollen foraging preferences of pollinators driven the evolution of such floral display patterns in pollen-only plants? This intriguing question presumes that pollinators foraging for pollen exhibit floral display preferences. However, to our knowledge, there is little or no quantitative information as to pollinator preferences for pollen-only plants. In this study, we asked whether pollen foraging bumble bees (Bombus impatiens) expressed congenital preferences for one pollen-only species over another. We further asked to what extent these preferences were shaped by floral experience, and if so, which parts of the flower were influenced by experience.

We used a pair of closely related and a pair of distantly related plant species that all offer only pollen as a reward. In Experiment 1, we explore the effect of experience collecting pollen from a single species on preference for flowers of that species relative to a second species either 1 or 24h later (absolute conditioning). In Experiment 2, via differential conditioning, we determine if the change in preference was due at least in part to receipt of a pollen reward. Finally, in Experiment 3, we assess the role of anthers and corolla in the formation of learned preference, using reciprocal combinations of these structures from 2 pollen-only species.

**METHODS**

**Subjects**

A total of 211 workers from 8 colonies of *B. impatiens* were used in experiments conducted between August 2014 and May 2015. Colonies were purchased from Koppert Biological Systems (Howell, MI) or from Biobest USA, Inc. (Wayzata, MN). Each experiment used approximately equal numbers of bees from at least 2 colonies.

Bees were allowed to forage daily for sucrose and pollen in either of 2 foraging arenas (82 cm × 60 cm × 60 cm and 82 cm × 60 cm × 30 cm). The arenas had clear acrylic ceilings and were lit from above by 40W and 60 Hz fluorescent lights (Lithonia Lighting). Lights were on a timer set to a 14:10 light-dark cycle. Colonies had access to ad libitum 2 M sucrose solution and pulverized honeybee-collected pollen (Koppert Biological Systems) within the foraging arena. Braided cotton wicks (6 inch Braided Cotton Rolls, Richmond Dental) that extended into 40 dram vials (BioQuip Products, Inc.) dispensed sucrose solution. Pollen was presented onchenille fibers, which were glued within 40 dram vials.

We used freshly clipped flowers from 4 *Solanum tridynamum*, 7 *Solanum elaegnifolium*, and 50 *E. affine* (a mix of Champion Blue, Little Champ Blue, and Royal Blue) in experiments. All species offer only pollen rewards, concealed within poricidal anthers, which are collected by certain bees, including *Bombus* species, using a behavior termed sonication. Sonication involves the generation of vibrations in order to extract pollen from flowers; the buzzing behavior is particularly strongly expressed when bees visit flowers bearing poricidal anthers (see Supplementary Video). *Solanum tridynamum* were purchased from a local museum (Arizona-Sonora Desert Museum, Tucson, AZ), *E. affine* were obtained commercially (Fred C. Gloeckner & Company, Inc., Harrison, NY), and *S. elaegnifolium* were obtained from wild collected samples (Jacob Francis, Roseville, CA). Plants were fertilized weekly (Miracle Gro, NPK = 15–30–15) and grown under natural light in a greenhouse with halogen lights used to extend day length to a 14:10 light-dark cycle. A total of 6136 flowers were used for experiments.

**General experimental protocol**

All training and testing took place in a foraging arena (L × W × H, 82 cm × 60 cm × 60 cm) painted gray on floor and sides to provide a neutral background. Bees were always individually trained and tested. To identify bees suitable for training and testing, 1–4 flower naive individuals were introduced into the arena simultaneously. When a bee landed on a flower in a training or test array, the others were removed from the arena immediately by catching them with vials and returned to the colony. Bees involved in training were labeled with unique color combinations of acrylic paint after the first training trial, before being returned to the colony.

During assays, a feeder containing 2 M sucrose solution was placed in the center of the foraging arena because some bees would not complete a trial without drinking (training phases: 24/104 bees; testing phases: 31/131 bees). To determine if sucrose feeding affected learning while foraging for pollen, we fed a set of bees on the same type of sucrose solution before the trial began and removed the arena feeder. Bees were fed by placing a feeder at the entrance to the flight arena and allowing the bees to crawl onto it and drink. Separately analyzing bees that fed from the arena feeder during training and those that did not feed in the arena revealed no obvious (and no statistically significant) differences in pollen foraging behavior (see Supplementary Materials).

Freshly clipped flowers were horizontally displayed (their natural orientation) on custom-built water tubes (Figure 2a), to prevent desiccation. The water tubes were Velcro mounted on a vertical array facing the flight chamber’s entrance. Flowers were arranged in a Cartesian grid (dimensions varying according to assay) with each water tube spaced 7 cm apart in the horizontal and vertical. For all experiments, fresh flowers were used for the training and testing phases for each bee. Flowers were never reused across trials or across bees.
In each experiment, we systematically alternated treatments that used the same species pairs in order to control for effects of day on behavior.

Behavioral assays

A bee was allowed to make a predetermined maximum number of successful visits in training and testing arrays, after which the trial was terminated and the bee was removed. A successful visit in this case was defined by the bee landing on a flower and engaging in sonication. A trial was also terminated if the bee did not forage on the array for a period of 5 min. However, virtually all bees reached the maximum number of allowed visits during training and testing. On completion of an assay, a bee was euthanized.

Video for all tests was captured at 30 fps with a high-definition digital camcorder (Canon VIXIA HF R400) positioned in front of the array. Audio was input to the camcorder using an external
microphone (33-3013 Lavalier Microphone, RadioShack) attached to the center of floral arrays. A Zoom H2 Handy Recorder (ZOOM Corporation) was used to amplify and verify sonication buzzes while trials were taking place.

We recorded 2 types of “events” during trials: landings with sonication buzzes (≈ “acceptances”) and landings without sonication buzzes (≈ “rejections”). A landing was defined as the bee touching the flower with at least 3 of its legs simultaneously. Sonication buzzes were identified by their distinctive sound and only occurred after a bee had landed. We used sonication buzzes as a proxy for pollen collection because sonication is the only behavior these bees use to extract pollen from the poricidal anthers. It is thus a consistent and reliable indicator of pollen collection. We included revisits, as defined by bees landing on the same target consecutively, in analyses (across all experiments, an average of 12.7% of landings were revisits). Additionally, we counted the number of unique flowers landed on in each array in all assays, to confirm that bees were visiting the majority of targets.

Experiments

Experiment 1: absolute conditioning

Here, we sought to describe patterns of initial and learned species preference. We assessed learned preference at different time points following training. We also asked whether the first and earliest choice by a bee predicted its overall pattern of preference within the array. This experiment used 120 bees from 7 colonies.

We assayed initial species preference by presenting 1 set of bees with arrays consisting of species pairs: either 10 S. tridynamum and 10 S. elaeagnifolium or 10 S. tridynamum and 10 E. affine. We did not use the third possible species pair (S. elaeagnifolium vs. E. affine) because preliminary results indicated that initially naive bees had a very strong preference for S. elaeagnifolium over E. affine, which would have made it difficult to analyze whether a shift in preference with experience on S. elaeagnifolium had occurred. We arranged the array such that species alternated with each position. Bees were allowed to make up to 40 acceptances. We discarded 1 bee that completed only 9.

We evaluated effects of experience with a different set of bees, using an absolute conditioning (S+) protocol (see Giurfa 2007 for a description of this protocol). Bees were first presented with 20 flowers of a single species (S. tridynamum, S. elaeagnifolium, or E. affine) in a vertical “training array” and allowed to make up to 40 acceptances. These bees were subsequently presented with a test array consisting of 10 flowers of the previously experienced species and 10 of the alternative species, with species alternated in the array by position. The test array consisted of either 10 S. tridynamum and 10 S. elaeagnifolium or 10 S. tridynamum and 10 E. affine flowers. Bees were allowed to make up to 40 acceptances in the test array.

To assay short-term effects of experience, 1 set of trained bees were allowed to enter the testing arena and forage 20-40 min after training. To assay long-term effects of experience, a different set of trained bees were allowed to enter the testing arena and forage approximately 24 h after training.

Experiment 2: differential conditioning

Here, we sought to determine if the changes in preference observed in Experiment 1 were due at least in part on receipt of a pollen reward. This experiment used 60 bees from 3 colonies.

We manipulated receipt of a pollen reward using glue. To create unrewarding flowers, drops of glue (Elmer’s Glue All, Elmer’s Products, Inc.) were applied to the tip of each porical anther with a clean toothpick and allowed to dry for 5 min. The glue sealed the anther pore, preventing release of pollen. In assays where rewarding flowers were used alongside unrewarding flowers, we controlled for possible effects of the glue on bee choice by applying drops of glue to the distal sides of each anther on a rewarding flower (without blocking the pores) and allowing the glue to dry for 5 min.

We first assayed preference by presenting 1 set of flower-naive bees using an array composed of 4 S. tridynamum and 4 E. affine flowers, all of which were unrewarding. Flowers were arranged in a 3 × 3 grid without a central flower (8 total targets), with species alternated by position. Bees were allowed to make up to 20 acceptances.

Using a different set of bees, we next employed a differential conditioning (S+/S−) protocol to assay effects of experience. Bees did not have access to sucrose solution. In the training phase, we first presented bees with a 2 × 2 array composed of flowers of a single species (either S. tridynamum or E. affine). Once a bee made a single acceptance, the array was removed from the arena and replaced with an array of the other species. We repeated these switches 6 times (3 presentations of each array). Switching arrays took approximately 20 s each time. Within a trial, we used the same arrays in each of the presentations, but after a bee had made 2 acceptances on a given flower, we replaced that flower with a fresh one to ensure that, in the case of rewarding targets, the anthers contained a sufficient amount of pollen. Using a tuning fork to vibrate the anthers, we subsequently verified that the rewarding flowers still had pollen after being visited. In each training sequence, we assigned one of the 2 species to be unrewarding. We alternated which species, the rewarding or unrewarding species, was presented first. For all 12 (of 48) bees that did not complete the training sequence, the S− had been presented first.

In the testing array, flowers were arranged in a 3 × 3 grid without a central flower (8 total targets), with species alternated by position. Bees were allowed to make up to 20 acceptances on this array. We made all flowers unrewarding. We did not use a rewarding test phase (as in the absolute conditioning assays/Experiment 1) because we wanted to eliminate the possibility that acquisition of a pollen reward in the test phase might alter or reinforce preference, even on the first floral visit, making it harder to assess whether experience in the training phase alone contributed to the bees’ learned preference.

Over the course of these assays, we sometimes observed that biting by the bees during sonication attempts broke open the sealed anthers (usually at the ventral base of the locules), causing pollen to be released. If this happened during training, the trial was discarded (1 out of 48 bees). If this happened during testing, we discarded all data subsequent to the point at which the anthers were opened.

Experiment 3: components of preference

Here, we sought to determine which part of the flower, corolla, and/or anthers, accounted for patterns of initial and conditioned preference. This experiment used 31 bees from 3 colonies.

Each of 4 types of targets used in this experiment was constructed from 2 freshly clipped flowers (Figure 2b). One flower had its anthers excised where the filament joined with the corolla (leaving the “corolla”). The other flower had its perianth mostly removed (Figure 2c), leaving a circle of corolla tissue to which the stamens, including their anthers, were joined. This circle of tissue was hot-glued into the center of the flower that had had its anthers removed. Four target types were produced in this way, 2 mosaic types and 2 sham controls: E. affine anthers glued to S. tridynamum corolla (mosaic 1), S. tridynamum anthers glued to E. affine corolla
(mosaic 2), E. affine anthers glued to E. affine corolla (sham control 1), and S. tridynamum anthers glued to S. tridynamum corolla (sham control 2). We did not observe any wilting or browning in these targets. Control assays comparing sham controls and intact flowers confirmed that cutting and gluing the tissue in this way did not affect bee behavior (see Supplementary Materials).

We assayed initial behavior by presenting 1 set of bees with 4 × 4 arrays of S. tridynamum and E. affine mosaics and sham controls. Bees were allowed to make up to 40 acceptances. Using a different set of bees, we assessed the short-term effects of experience with S. tridynamum or E. affine on mosaics and sham control flowers by first training and then testing the bees. During training, bees were presented with a 4 × 5 array of intact flowers of a single species (S. tridynamum or E. affine) and allowed to make up to 40 acceptances. Subsequently, bees were tested with a 4 × 4 array consisting of equal numbers of each sham and mosaic targets 20–40 min after training. Bees were allowed to make up to 40 acceptances in the test. Targets of different types were assigned to positions such that all position–target–type combinations were equally represented across all trials and no single type of target appeared more than once in a row or column within a given array.

Data analyses

All data were analyzed using R v.3.2.0 (R Development Core Team 2010). We used landings for measures of preference. We used approaches only to assess whether cutting flowers and gluing different tissues together affected behavior.

We used a binomial test to analyze whether naive bees had a preference for one or the other species with their first landing choice. To analyze preference across landings for naive bees that visited unrewarding S. tridynamum/E. affine arrays, we used a paired t-test.

To analyze the effect of experience on species preference, we used binomial generalized linear mixed effect models (GLMERs), specifying type II Wald chi-square (χ²) tests via the Anova() function in the car package (Fox 2015). For these models we included “BeeID” as a random factor and visits as repeated measures within BeeID and the fixed effects “species choice” (S. tridynamum or S. elaegnifolium; S. tridynamum or E. affine) and “treatment” (control, S. tridynamum, or S. elaegnifolium; control, S. tridynamum, or E. affine). GLMERs were carried out using the glmer() function in the lme4 package (Bates et al. 2015). In cases of significant effects, we ran Tukey’s post hoc test using the glht() function in the multcomp package (Hothorn et al. 2015) to determine which pairs were significant.

For all GLMERs, maximal models were run first. For each analysis, we performed 2 rounds of backward elimination (as described in Fox 2015). We checked first whether any interaction terms should be eliminated from the model and then whether any main effects should be removed. We used the anova() function in R to examine significance for each of these effects relative to the full model.

To analyze potential interactions between corolla and anther types in the components of preference assay (Experiment 3) and between treatment order and preference in the differential conditioning assay (Experiment 2), we used mixed multinomial logit models (MMNLMs). For the components of preference model, we included “BeeID” as a random factor and the fixed factors “treatment” (S. tridynamum and E. affine and control), “anther choice” (S. tridynamum or E. affine), and “corolla choice” (S. tridynamum or E. affine). We also ran MMNLMs for each treatment separately, to examine interactions within a treatment. For the differential conditioning model, we included “BeeID” as a random factor and the fixed factors “species choice” (S. tridynamum or E. affine), “training treatment” (S. tridynamum or E. affine), and “treatment order” (S. tridynamum or E. affine). MMNLMs were carried out using the mlogit() function in the mlogit package (Henningsen and Toomet 2011; Croissant 2012).

RESULTS

Bees learn preferences and these learned preferences persist for at least 24 h

In the absolute conditioning assay (Experiment 1), bees given experience foraging for pollen on S. tridynamum or S. elaegnifolium and then tested in an S. tridynamum/S. elaegnifolium array after 20–40 min (short-term retention test) or after 1 day (long-term...
behavioral ecology retention test) preferred the experienced flower type (Figure 3a; GLMER overall effect for the short-term retention test: type II Wald $\chi^2$ tests for experience x species choice: $\chi^2 = 30.688$, degrees of freedom [df] = 2, $P < 0.0001$; Figure 3b; GLMER overall effect for the long-term retention test: type II Wald $\chi^2$ tests for experience x species choice: $\chi^2 = 14.659$, df = 2, $P < 0.0007$).

Likewise, bees that were given experience foraging on $S$. tridynamum or $E$. affine and then tested in an $S$. tridynamum/$E$. affine array after 20–40min short-term retention test) or after 1 day long-term retention test) also preferred the experienced flower type (Figure 3c; GLMER overall effect for the short-term retention test: type II Wald $\chi^2$ tests for experience x species choice: $\chi^2 = 30.154$, df = 2, $P < 0.0001$; Figure 3d; GLMER overall effect for the long-term retention test: type II Wald $\chi^2$ tests for experience x species choice: $\chi^2 = 28.607$, df = 2, $P < 0.0001$).

Naive bees did not express preferences for one pollen-only species over another in any experiment.

For this analysis, we pooled data for naive bees from the same species pairings within Experiment 1. This was done so that we

![Figure 3](https://academic.oup.com/beheco/article-abstract/27/3/731/2365383/736)

**Figure 3**
Top panels: species preference for initially naive and experienced bees visiting arrays consisting of an equal number of rewarding $S$. tridynamum and $S$. elaeagnifolium in either (a) the short-term or (b) long-term retention tests. $N$ = 14 and 10 for initially naive bees in the short-term and long-term retention tests, respectively. $N$ = 10 for each experienced treatment, aside from $N$ = 13 for bees given experience on $S$. tridynamum in the long-term retention test. Middle panels: species preference for initially naive and experienced bees visiting arrays consisting of an equal number of rewarding $S$. tridynamum and $E$. affine in either (c) the short-term or (d) long-term retention tests. $N$ = 12 for initially naive bees. $N$ = 10 for each experienced treatment. Bottom panel: (e) species preference for naive and experienced bees visiting arrays consisting of an equal number of unrewarding $S$. tridynamum and $E$. affine. $N$ = 18 for each experienced treatment. $N$ = 12 for the naive treatment. Letters above bars within a panel indicate significant differences at $P < 0.05$ according to a Tukey’s post hoc test.
could achieve a large enough sample size to analyze differences in naive preference with a binomial test. There was no significant difference in the number of naive bees that made their first landing choice on *S. tridynamum* versus *E. affine* in the rewarding or unrewarding *S. tridynamum*/*E. affine* arrays (Experiments 1 and 2, respectively) (% bees that made their first landing on *S. tridynamum*: absolute conditioning, 50.0%; differential conditioning, 41.7%; binomial test: absolute conditioning, \( P > 0.185, N = 18 \); differential conditioning, \( P > 0.193, N = 12 \)). There was also no significant difference in the number of naive bees that made their first landing choice on *E. affine* when foraging in the rewarding *S. tridynamum*/*E. affine* array (Experiment 1) (% bees that made their first landing on *S. tridynamum*: 40.0%; binomial probability: \( P > 0.097, N = 25 \)). Additionally, naive bees that visited the unrewarding *S. tridynamum*/*E. affine* array in the differential conditioning assay (Experiment 3) showed no significant difference in preference across all landings for either species (proportion of landings to *S. tridynamum* × *E. affine*: 57.5%; paired \( t \)-test: \( t_{11} = 1.236, P > 0.242 \)).

**Conditioned preference is influenced by receipt of pollen**

In the differential conditioning (S+/S−) assay (Experiment 2), bees that were trained to either *S. tridynamum* or *E. affine* and then tested in an unrewarding *S. tridynamum*/*E. affine* array after 20–40 min preferred the flower type that was rewarding in the training phase, relative to bees that were trained on the alternative flower type. Additionally, bees that were trained on *E. affine*, but not those trained to *S. tridynamum*, preferred the previously rewarded flower type relative to naive bees (Figure 3c; GLMER overall effect: type II Wald \( \chi^2 \) tests for experience × species choice: \( \chi^2 = 14.659, \text{df} = 2, P < 0.0007 \)).

**Learned preferences were mediated by both anther and corolla, but learned preferences for the anther were much stronger**

In the components of preference assay (Experiment 3), bees that were given experience on *S. tridynamum* or *E. affine* and then tested in an *S. tridynamum*/*E. affine* mosaic/sham array after 20–40 min preferred the anthers of the experienced flower species (Figure 4a; GLMER overall effect: type II Wald \( \chi^2 \) tests for experience × anther species choice: \( \chi^2 = 49.512, \text{df} = 2, P < 0.0001 \)).

These same bees preferred the corollas of the experienced flower species significantly more than bees that were given experience on the alternative flower species, but not significantly more than initially naive bees (Figure 4b; GLMER overall effect: type II Wald \( \chi^2 \) tests for experience × corolla species choice: \( \chi^2 = 15.441, \text{df} = 2, P < 0.0005 \)).

**Anther and corolla generally did not interact to affect preference**

Across the 3 experience treatments, in Experiment 3, bees preferred the anthers and corolla of the experienced flower type; there was no overall interaction between the effects of anther and corolla species on preference (MMNLM: flower choice × anther species: \( t = -16.388, P < 0.0001 \); flower choice × corolla species: \( t = -3.242, P < 0.002 \); flower choice × anther species:corolla species: coefficient estimate = 0.224, \( t = 0.669, P = 0.503 \)). Nevertheless, there was 1 specific interaction: Bees given experience on *E. affine* and then tested in the *S. tridynamum*/*E. affine* mosaic/sham array exhibited less of a preference for the species identity of the corolla when choosing flowers with *E. affine* anthers, than when choosing flowers with *S. tridynamum* anthers (MMNLM: coefficient estimate = −0.572, \( t = 2.201, P < 0.028 \)).

**DISCUSSION**

Pollen foraging bumble bees displayed modest innate preferences for certain pollen-only plant species, but formed strong, lasting preferences for even initially less-preferred species after experience collecting pollen from them. Although naive preferences were weak, our results nevertheless indicate that they can have significant effects on what bees learn to prefer. In short, both innate and learned components of preference that have been demonstrated for nectar foraging also appear to characterize pollen foraging. Both components of preference may thus direct the evolution of floral display traits in any plant species where pollinators collect pollen in exchange for the service of its transfer (Schiestl and Johnson 2013).

Although several studies have shown that bees can learn floral color cues in association with pollen rewards or related facsimiles (Grüter et al. 2008; Nicholls and Hempel de Ibarra 2014; Muth et al. 2015, 2016), the present study is the first, to our knowledge, to characterize effects of experience on preference for live plants in a pollen foraging context. Notably, even in a nectar foraging context, using live plants to study learned preference under controlled

Figure 4

(a) Anther or (b) corolla preference for each species, for naive and experienced bees visiting arrays consisting of an equal number of *Solanum tridynamum* and *Exacum affine*. \( N = 10 \) for each treatment, save for bees given experience on *E. affine* where \( N = 11 \). Letters above bars within a panel indicate significant differences at \( P < 0.05 \) according to a Tukey’s post hoc test.
conditions is relatively uncommon (e.g., Schemske and Bradshaw 1999; Cane 2011; Dobson et al. 2012). One reason may be that the use of whole plants limits inferences as to mechanisms underlying patterns of floral preference. For instance, associative learning is an obvious candidate for the mechanism underlying our results, with the pollen stimulus serving as an unconditioned stimulus with which floral scent or color are paired as conditioned stimuli. However, the unconditioned stimulus could alternatively consist of 1 or more stimuli associated with the anthers or even other flower parts. Along these lines, our results from Experiment 1 suggest that even an unrewarding experience can positively influence subsequent preference for that species in a setting where both options are equally available (Supplementary Figure S2). Similarly, the floral display typically consists of multicomponent signals (Raguso 2004; Leonard et al. 2011b). At present, we have not precisely defined which of these might represent the conditioned stimulus or stimuli. Yet pollinator preferences for live plants bearing live flowers are what directly influence floral evolution. Without examining the features that pollinators might associate with pollen rewards offered by real flowers, characterizing the floral cues learned by pollinators completely would be difficult.

By using live flowers, we uncovered new information about the display traits involved in learned preferences for pollen-only plant species. We showed that both corolla and anther responses were modified by experience, although anther responses were modified significantly more. Additionally, learned preference in one instance was mediated through an interaction between anther and corolla type. Our findings are consistent with those of recent studies of color learning in artificial flowers offering pollen as rewards (Muth et al. 2015). Muth et al. demonstrated that bees can associate either corolla color or anther color with the presence of free pollen on artificial anthers and can even learn specific combinations of corolla and anther color. Their studies offer good evidence for the role of associative learning in this process (see also Grütter et al. 2008; Arenas and Farina 2012; Nicholls and Hempel de Ibarra 2014).

Our results also point to possible differences in the display traits used by nectar foraging versus pollen foraging pollinators. Features of the corolla are key cues for nectar foragers. For instance, to direct pollinators to hidden nectaries, many plants display prominent guides that can differ from the corolla with respect to texture, color, shape, and size (e.g., Kevan and Lane 1985; Dafni and Kevan 1996; Hansen et al. 2012). Although corollas of certain pollen-only flowers feature putative pollen guides (e.g., Eschscholzia poppies), we might expect androecial cues to be especially important for pollen foragers, as found here. Our findings are not entirely surprising because visual (Lunau 1995) and olfactory (Dobson et al. 1990; Bergström et al. 1995; Dobson et al. 1996) components of pollen are known to be distinct from those of the corolla and have been suggested to play an important role in pollinator behavior (Lunau 1995; Dobson et al. 1999; Goulson et al. 2001). However, when pollen is concealed—as in species with poricidal anthers like those used in the present study—pollen odor might play little to no role in pollinator attraction (Buchmann and Cane 1989; Burkart et al. 2013; Russell A, Leonard A, Papaj D, unpublished data). Instead, for angiosperm species that offer concealed pollen rewards, other floral features closely associated with these rewards, such as the color or scent of anther tissue, might play a more important role. Consistent with this perspective, experiments in progress suggest that anther chemistry plays a more important role than pollen chemistry in eliciting landings by bees on S. trifidum (Russell A, Papaj D, unpublished data).

What are the implications of the pollen foraging preferences characterized here for the evolution of floral morphology and chemistry? Although a rigorous accounting of the pattern of floral rewards among angiosperm species is wanting, a substantial fraction of species offer pollen as a floral reward (Vogel 1978; Faegri 1986). Potentially tens of thousands of species, perhaps even hundreds of thousands of species, have floral displays that have evolved to reflect in part the impact of pollinator behavior in the context of pollen collection. For those species that offer only pollen rewards, floral evolution will surely be influenced very strongly by preference in the context of pollen collection.

Indeed, many pollen-only species show patterns of floral morphology distinct from those of nectar-bearing species. In particular, pollen-only species in 17 plant families show a convergent pattern of floral morphology termed the solanoid flower form (Faegri 1986; De Luca and Vallejo-Marín 2013 and references within). A solanoid flower consists of a radially symmetrical corolla with anthers that form a “bulls-eye” in the center of the corolla (Figure 1). Although the corolla is typically (human) blue or purple, the anthers are often (human) yellow. The typical solanoid flower’s anthers have a poricidal morphology, in which the pollen is concealed inside the tube-like anthers, and must be extracted through pores or slits in the tip of the anther through sonication (Vogel 1978; Faegri 1986).

Because bees are nearly the only pollinators that engage in sonication (Pellmyr 1985; Buchmann and Cane 1989), their behavior has likely been a major force in convergence and divergence relating to this floral “syndrome” (Buchmann 1983; De Luca and Vallejo-Marín 2013). For example, bees’ preferences among center-surround color contrast (Lunau 1991; Lunau 1992b; Lunau et al. 2006) may have selected for these patterns in solanoid flowers. Accordingly, corolla cues might function to attract pollen foragers, as they do for nectar foragers visiting nectariferous species (e.g., Kevan and Lane 1985; Dafni and Kevan 1996; Hansen et al. 2012), and also to direct foragers to the anthers. At short range, anther cues may become more detectable, and because they are the source of pollen, bees on or near flowers should attend strongly to such cues. Our results provide strong evidence for the potency of anther cues and how it is strengthened by experience. Despite their convergence in form, plant species with solanoid flowers show variation in visual respects (Figure 1) and surely vary as well in floral cues such as scent and microtexture. Because all of the species we studied had solanoid flower morphology, our findings about preference patterns are especially pertinent to understanding differences among solanoid species, as they suggest that variation in corolla and anther features could reflect bee preferences.

In over a century of research on pollinator preference and floral trait evolution, we have barely scratched the surface of this subject in relation to pollen collection. In the present study, we find strong evidence for the formation of learned preferences. Bees foraging for pollen from real flowers show 1) weak initial floral preferences, 2) the capacity to rapidly modify and, in some circumstances, magnify these preferences with experience, 3) long-term retention of the effects of experience, and 4) changes with experience that are best explained as associative learning. Our evidence suggests that both corolla and anther responses are involved in learned preferences, with anther responses strongly influenced by experience. Yet to be determined is the extent to which congenitally expressed preferences reflect exploitation of receiver biases, such as have been put forward in connection with nectar-bearing and rewardless plant species (Schiestl and Johnson 2013).
SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/

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Data accessibility: we will archive data for this project at Dryad on acceptance.

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REFERENCES

 Arenas A, Farina WM. 2012. Learned olfactory cues affect pollen-foraging preferences in honeybees, Apis mellifera. Anim Behav. 83:1023–1033.

 Bates D, Maechler M, Bolker B, Walker S. 2015. lme4: linear mixed-effects models using Eigen and S4. R package version 1.1-9 [cited 2015 Nov 8]. Available from: http://CRAN.R-project.org/package=lme4.

 Bergström G, Dobson HEM, Groth I. 1993. Spatial fragrance patterns within the flowers of Ranunculus acris (Ranunculaceae). Plant Syst Evol. 195:221–242.

 Bond AB, Kamal AC. 2005. Spatial heterogeneity, predator cognition, and experimental trials. Proc Natl Acad Sci USA. 103:3214–3219.

 Buchmann SL. 1983. Buzz pollination in angiosperms. In: Jones CE, Little RJ, editors. Handbook of experimental pollination biology. New York: Van Nostrand Reinhold, p. 73–113.

 Buchmann SL, Cane JH. 1989. Bees assess pollen returns while sonicking Solanum flowers. Oecologia. 81:289–294.

 Burkart A, Schlindwein C, Lanau K. 2013. Assessment of pollen reward and pollen availability in Solanum stenomphallum and Solanum panamitanum for buzz-pollinating carpenter bees. Plant Biol. 16:503–507.

 Cane JH. 2011. Specialist Osmia bees forage indiscriminately among hybridizing Balsamorhiza floral hosts. Oecologia. 167:107–116.

 Chittka L. 1992. The color hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of color opponency. J Comp Physiol A. 170:533–543.

 Chittka L, Thomson JD. 2005. Cognitive ecology of pollination: animal behaviour and floral evolution. Cambridge (UK): Cambridge University Press.

 Clarke D, Whitney H, Sutton G, Robert D. 2013. Detection and learning of floral electric fields by bumblebees. Science. 340:66-69.

 Croissant Y. 2012. Estimation of multimodal logit model in R: the package mlogit: R package version 0.2-3 [cited 2015 Nov 8]. Available from: http://CRAN.R-project.org/package=mlogit.

 Dafni A, Kevan PG. 1996. Floral symmetry and the nectar guides: ontogenetic constraints from floral development, colour pattern rules and functional significance. J Linn Soc Bot. 120:371–377.

 Dobson HM, Ayasse M, O’Neal KA, Jacka JA. 2012. Is flower selection influenced by chemical imprinting to larval food provisions in the generalist bee Osmia bicolor (Megachilidae)? Apidologie. 43:698–714.

 Dobson HEM, Bergström G, Groth I. 1990. Differences in fragrance chem in the nectar guides to bees and plants. Funct Ecol. 1991:929–1301.

 Dobson HEM, Bergström G, Groth I. 1996. Pollen advertisement: chemical contrasts between whole-flower and pollen odors. Am J Bot. 83:877–885.

 Faegri K. 1986. The solanoid flower. Trans Bot Soc Edinburgh. 35:375–403.

 Foster JJ, Sharkey CR, Gaworska VA, Roberts NW, Whitney HM, Partridge JC. 2014. Bumblebees learn polarization patterns. Curr Biol. 24:1413–1420.

 Fox J. 2015. Applied regression analysis and generalized linear models. 3rd ed. London: Sage Publications, Inc.

 Fukushi T. 1989. Learning and discrimination of coloured papers in the walking blowfly, Lucilia cuprina. J Comp Physiol A. 166:57–64.

 Giger AD, Srinivasan MV. 1995. Pattern recognition in honeybees: eidetic imagery and orientation discrimination. J Comp Physiol A. 176:791–793.

 Giurfa M. 2007. Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. J Comp Physiol A. 193:801–824.

 Goulson D, Chapman JW, Hughes WHO. 2001. Discrimination of unrewarding flowers by bees: direct detection of rewards and use of repellent scent marks. J Insect Behav. 14:669–678.

 Grabowska JH, Kimbro DL. 2005. Predator-avoidance behavior extends trophic cascades to refuge habitats. Ecology, 86:1312–1319.

 Grüter C, Arenas A, Farina WM. 2008. Does pollen function as reward for honeybees in associative learning? Insect Soc. 55:425–427.

 Gumbert A. 2000. Color choices by bumble bees (Bombus terrestris): innate preference and generalization after learning. Behav Ecol Sociobiol. 48:36–43.

 Hansen DM, Van der Niet T, Johnson SD. 2012. Floral signposts: testing the significance of visual ‘nectar guides’ for pollinator behavior and plant fitness. Proc Biol Sci. 279:634–639.

 Henningsen A, Toomet O. 2011. maxLik: a package for maximum likelihood estimation in R. Comput Stat. 26:443–458.

 Hopkins R, Rausher MD. 2012. Pollinator-mediated selection on flower color allele drives reinforcement. Science. 333:1090–1092.

 Holthorn T, Brest T, Westfall P, Heuberger RM, Scheutzeuner A, Schieb S. 2015. Simultaneous inference in general parametric models. R package: version 1.4-1 [cited 2015 Nov 8]. Available from: http://CRAN.R-project.org/package=multcomp.

 Hurty TA, Healy SD. 2002. Cue learning by rufous hummingbirds (Selasphorus rufus). J Exp Psych. 28:209–223.

 Jalvingh KM, Chang PL, Nuzhdin SV, Wertheim B. 2015. Genomic changes under rapid evolution: selection for parasitoid resistance. Proc Biol Sci. 282:20132303.

 Kelber A, Pfaff M. 1997. Spontaneous and learned preferences for visual flower features in a diurnal hawkmoth. Israel J Plant Sci. 45:235–245.

 Kevan PG, Baker HG. 1983. Insects as flower visitors and pollinators. Annu Rev Entomol. 28:407–453.

 Kevan PG, Lane MA. 1982. Flower petal microtexture is a tactile cue for bees. Proc Natl Acad Sci USA. 82:4750–4752.

 Kitaoka TK, Nieh JC. 2009. Bumble bee pollen foraging regulation: role of pollen quality, storage levels, and odor. Behav Ecol Sociobiol. 63:500–510.

 Leonard AS, Dornhau A, Papaj DR. 2011a. Flowers help bees cope with uncertainty: signal detection and the function of complex floral signals. J Exp Biol. 214:113–121.

 Leonard AS, Dornhau A, Papaj DR. 2011b. Why are floral signals complex? An outline of functional hypotheses. In: Patiny S, editor. Evolution of plant-pollinator relationships. Cambridge (UK): Cambridge University Press. p. 279–300.

 Leonard AS, Masek P. 2014. Multisensory integration of colors and scents: insights from bees and flowers. J Comp Physiol A. 200:463–474.

 Leonard AS, Papaj DR. 2011. ‘X’ marks the spot: the possible benefits of nectar guides to bees and plants. Funct Ecol. 25:1293–1301.

 Lewis AC. 1993. Learning and the evolution of resources: pollinators and flower morphology. In: Papaj DR, Lewis AC, editors. Insect learning: ecological and evolutionary perspectives. New York: Chapman & Hall. p. 219–242.

 De Luca PA, Vallejo-Marín M. 2013. What’s the “buzz” about? The ecology of buzz-pollinating carpenter bees. Plant Biol. 15:429–435.

 Lanau K. 2011. Innate flower recognition in bumblebees (Bombus terrestris, B. lucorum; Apidae): optical signals from stamens as landing reaction receptors. Ethology. 88:203–214.

 Lanau K. 1992a. Limits of colour learning in a flower-visiting hoverfly, Eristalis tenax (L.; Syrphidae, Diptera). Euro J Neurosci. 5 (Suppl.):103.

 Lanau K. 1992b. A new interpretation of flower guide colouration: absorption of ultraviolet light enhances colour saturation. Plant Syst Evol. 243:51–65.

 Lanau K. 1993. Notes on the colour of pollen. Plant Syst Evol. 198:235–252.
Lunau K, Fieselmann G, Heuschen B, van de Loo, A. 2006. Visual targeting of components of floral colour patterns in flower-naive bumblebees (Bombus terrestris; Apidae). Naturwissenschaften. 93:325–328.
Luo SX, Chaw SM, Zhang D, Renner S. 2010. Flower heating following anthesis and the evolution of gall midge pollination in Schisandraceae. Am J Bot. 97:1220–1228.
Mallet J, Joron M. 1999. Evolution of diversity in warning color and mimicry: polymorphism, shifting, balance, and speciation. Annu Rev Ecol Syst. 30:201–233.
Menzel R. 2012. The honeybee as a model for understanding the basis of cognition. Nat Rev. 13:758–768.
Morse DH. 1982. The turnover of milkweed pollinia on bumble bees, and implications for outcrossing. Oecologia. 53:187–196.
Muth F, Papaj DR, Leonard AS. 2015. Bees remember flowers for more than one reason: pollen mediates associative learning. Anim Behav. 111:93–100.
Muth M, Papaj DR, Leonard AS. 2016. Colour learning when foraging for nectar and pollen: bees learn two colours at once. Biol Lett. 11:20150628.
Nicholls E, Hempel de Ibarra N. 2014. Bees associate colour cues with differences in pollen rewards. J Exp Biol. 217:2783–2788.
Nicolson SW, van Wyk JH. 2011. Bee food: the chemistry and nutritional value of nectar, pollen and mixtures of the two. Afr J Zool. 46:197–204.
Pellmyr O. 1985. Pollination ecology of Cimicifuga arizonica (Ranunculaceae). Bot Gaz. 146:404–412.
Paseo RA. 2004. Flowers as sensory billboards; progress towards an integrated understanding of floral advertisement. Curr Opin Plant Biol. 7:433–440.
R Development Core Team. 2010. R: a language and environment for statistical computing. Vienna (Austria): R Foundation for Statistical Computing.
Salch N, Chittka L. 2006. The importance of experience in the interpretation of conspecific chemical signals. Behav Ecol Sociobiol. 61:215–220.
Schemske DW, Bradshaw HD Jr. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (Mimulus). Proc Natl Acad Sci. 96:11910–11915.
Schiestl FP, Johnson SD. 2013. Pollinator-mediated evolution of floral signals. Trends Ecol Evol. 28:307–315.
Seymour RS, Matthews PGD. 2006. The role of thermogenesis in the pollination biology of the Amazon waterlily Victoria amazonica. Ann Bot. 98:1129–1135.
Simpson BB, Neff JL. 1981. Floral rewards: alternatives to pollen and nectar. Ann Mo Bot Gard. 68:301–322.
Skorupski P, Chittka L. 2010. Differences in photoreceptor processing speed for chromatic and achromatic vision in the bumblebee, Bombus terrestris. J Neurosci. 30:3896–3903.
Vogel S. 1978. Evolutionary shifts from reward to deception in pollen flowers. In: Richards AH, editor. The pollination of flowers by insects. London: Academic Press. p. 89–96.
Waser NM, Ollerton J. 2006. Plant–pollinator interactions: from specialization to generalization. Chicago (IL): The University of Chicago Press.
Weiss MR. 1997. Innate colour preferences and flexible colour learning in the pipevine swallowtail. Anim Behav. 53:1043–1052.
Whitney HM, Chittka L, Bruce TJA, Glover BJ. 2009. Conical epidermal cells allow bees to grip flowers and increase foraging efficiency. Curr Biol. 19:948–953.
Whitney HM, Kolle M, Andrew P, Chittka L, Steiner U, Glover BJ. 2009. Floral iridescence, produced by diffractive optics, acts as a cue for animal pollinators. Science. 323:130–133.
Wilms J, Eltz T. 2007. Foraging scent marks of bumblebees: footprint cues rather than pheromone signals. Naturwissenschaften. 95:149–153.