



Linking components of complex signals to morphological part: the role of anther and corolla in the complex floral display

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ARTICLE INFO

Article history:

Received 7 July 2017

Initial acceptance 29 August 2017

Final acceptance 18 October 2017

MS. number: A17-00541R

Keywords:

bumblebee
communication
complex signal
floral display
floral morphology
learning
multimodal cue
pollen
poricidal anther
signal interaction

Signals used in communication are frequently complex, being composed of multiple signal components that in combination improve information transfer. A variety of morphological parts are typically used to transmit components of any given complex signal. Our understanding of why a given morphological part is used to transmit a given signal component is poor. We hypothesized that the function of a given signal component is improved by its association with its morphological part and that such parts interact functionally to transmit information. In a laboratory study we characterized the function of different floral signal components transmitted by associated floral parts and the interaction of those signal components. Using *Solanum houstonii* flowers, we focused on two major floral parts, corolla and anthers, involved in signalling bumblebee, *Bombus impatiens*, visitors. We further examined how experience affected the relationship between signal component and floral part. Floral visits involve a stepwise process in which bees approach, land and acquire pollen. We found that the corolla plays the dominant role in eliciting approaches by bees, whether naïve or experienced. Landing is elicited by corolla signals and, to a lesser but additive degree, anther signals. Following experience, anther signals nearly completely dominate corolla signals in eliciting landing. The anthers convey signals mediating pollen acquisition, regardless of the bee's experience level. Our findings suggest there is selection for specific relationships between signal components and morphological parts, which in turn might drive complex signal evolution.

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Organisms frequently communicate using complex signals (i.e. displays; Hebets & Papaj, 2005; Hebets et al., 2016) composed of multiple interacting components in one or more sensory modalities (e.g. visual, olfactory, tactile; Hebets & Papaj, 2005; Herberstein, Wignall, Hebets, & Schneider, 2014; Kaczorowski, Leonard, Dornhaus, & Papaj, 2012). A variety of morphological parts may be involved in transmitting a complex signal. For instance, the complex mating display of many spider species involves multiple seismic and visual cues produced by the forelegs and the abdomen (e.g. Elias, Sivalingham, Mason, Andrade, & Kasumovic, 2010; Girard, Kasumovic, & Elias, 2011; Hebets & Uetz, 1999). Selection presumably acts to maintain the complex signal (Hebets & Papaj, 2005; Leonard, Dornhaus, & Papaj, 2011) and by extension the morphological parts responsible for sending the complex signal.

While a great deal of attention has been given to characterizing the benefits of complex signals (see Hebets et al., 2016; Leonard et al., 2011; Leonard, Dornhaus & Papaj, 2012), less is known about why particular morphological parts are used for particular signal components. A given morphological part might improve the function of its associated signal component. For instance, tadpole 'tail spots' and lycaenid butterfly 'false head' visual signals capture predator attention, and because these signals are transmitted by the tails, they direct attacks to the tail: an expendable region of the body (Sourakov, 2013; Van Buskirk, Aschwanden, Buckelmüller, Reolon & Rüttiman, 2004). Likewise, signal components that differ in their function may be segregated to some extent among physical parts that improve those different functions. For example, visual and acoustic cues associated with the upper train of peacocks attract females from a distance while the lower train provides only visual cues used in close-range courtship (Yorzinski, Patricelli, Babcock, Pearson, & Platt, 2013). The upper train is highly conspicuous relative to the lower train, and this difference in conspicuousness potentially improves each morphological part's

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signalling function. Such parts might conceivably act independently of one another or interact to some degree to improve the function of a complex signal.

Biotically pollinated plants typically communicate with their pollinators via floral displays, composed of tactile, visual, olfactory, humidity, and even electrical cues (Clarke, Whitney, Sutton, & Robert, 2013; Giger & Srinivasan, 1995; Foster et al., 2014; Muth, Papaj, & Leonard, 2016; von Arx, Goyret, Davidowitz, & Raguso, 2012; Whitney et al., 2009; Whitney, Chittka, Bruce & Glover, 2009). These cues form a complex signal that can benefit both plant and pollinator (Leonard et al., 2011, 2012). Floral cue composition commonly differs among morphologically distinct floral parts (Leonard et al., 2011, 2012; Fig 1d–f). For example, a flower's corolla often displays different colour patterns than its anthers (e.g. Fig 1). Likewise, the anthers of many plant species produce different and more kinds of odours than the corolla (Burdon, Raguso, Kessler, & Parachnowitsch, 2015; Dobson, Groth, & Bergström, 1996). Pollinators should be able to perceive and respond to such differences (e.g. Ashman, Bradburn, Cole, Blaney, & Raguso, 2005; Guerrieri, Schubert, Sandoz, & Giurfa, 2005; Muth

et al., 2016; Riffell et al., 2008). Given that the signal components of different floral parts may differ in sensory modality, do the different floral parts serve different functions for the complex floral signal? Do the signal components in different physical parts act independently, or do they interact? These questions are largely unanswered in the existing literature (but see Ashman, Swetz, & Shivitz, 2000; Connolly & Anderson, 2003; Lunau, 1992; Ushimaru et al., 2007).

Several basic steps are required for pollination, which involves the transmission of pollen to the pollinator and typically involves the acquisition of a floral reward by the pollinator. Plants must initially provide signal components that attract pollinators to the flowers (Fig 1a). Following pollinator attraction, signal components that orient the pollinator on the flower come into effect (Fig 1b). For instance, many flowering plant species display so-called nectar guides on their corollas that direct pollinators to nectaries, which hold the nectar reward (Leonard & Papaj, 2011; Penny, 1983). Finally, signal components that facilitate pollen transfer to the pollinator come into effect (Fig 1c). For plant species that offer pollen as a floral reward to their pollinators (Kevan & Baker, 1983;

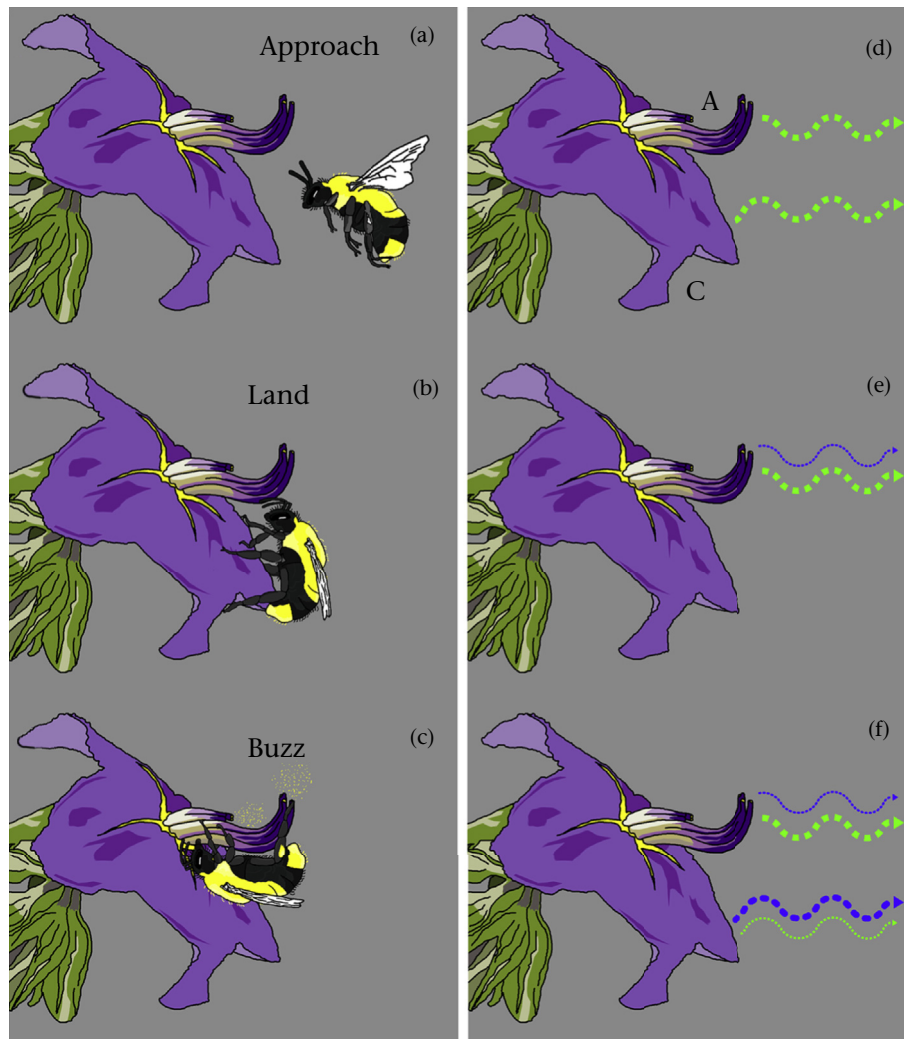


Figure 1. The basic steps involved in the transfer of pollen to the bee from flowers that offer pollen as the sole reward, such as those of *Solanum houstonii*. (a) Attraction is followed by (b) landing, which is followed by (c) physical contact with the anthers and then pollen extraction (via buzzing). The complex floral signal might be transmitted by (d) multiple floral parts (such as the anthers, A, and the corolla, C), (e) multiple signal components from a single floral part, or even (f) multiple signal components each from a separate floral part. Likewise, each basic step involved in the transfer of pollen might involve different combinations and weighting of floral parts and their signal components. Separate signal components are indicated by differently coloured lines; line thickness indicates weighting.

Nicolson, 2011; Simpson & Neff, 1981), such signal components might conceivably elicit extraction of pollen from the pollen-bearing anthers.

In this study we sought to determine which morphological parts, anthers or corolla, are involved in the steps leading to acquisition of a pollen reward by a pollinator and how the floral parts interact. We used flowers of a pollen-only species, *Solanum houstonii*, and pollen foraging bumblebees, *Bombus impatiens*. Like bee-pollinated species in at least 17 families of flowering plants, *S. houstonii* exhibits a convergent pattern of floral morphology called the solanoid flower form (De Luca & Vallejo Marin, 2013; Faegri, 1986; see Russell & Golden, 2016 for images). Solanoid flowers resemble a bullseye; the typically blue or purple (to the human eye) corolla is reflexed away from the anthers, exposing a central grouping of enlarged tubelike anthers ('poricidal anthers'; Buchmann, 1983) that are typically yellow to the human eye. We hypothesized that cues that allow bumblebees to first find and then select flowers, as well as locate and extract the sole floral reward (pollen), would be segregated between the corolla and anther and that the two morphological parts would interact to mediate bee foraging. Finally, we examined the role of experience in shaping bee responses to floral parts and cues. To achieve these goals, we isolated the two floral parts and their respective visual and olfactory cues by manipulating live flowers in a series of laboratory experiments.

METHODS

Outline of Study

We conducted three laboratory experiments between May 2014 and August 2015 in a University of Arizona laboratory. The first two experiments each had two treatments (one no-experience treatment and one flower-experience treatment), while the third experiment had three treatments (one additional no-experience treatment). The no-experience treatments involved an assessment of responses by flower-naïve bees to test flowers that had been modified to isolate the floral parts or their signal components. To evaluate strictly the floral signals influencing bee attraction, landing and behaviour leading to pollen collection, all test flowers were manipulated such that pollen could not be collected from them. In the flower-experience treatments, flower-naïve bees were given a set amount of experience collecting pollen from intact flowers in a training phase. To evaluate how bee responses changed in response to training, in the subsequent testing phase these bees were allowed to forage in an array identical to that in which bees in the no-experience treatment foraged. The no-experience treatment evaluates innate responses in terms of what flower types bees first visited, and also the overall responses resulting beyond what was observed in first visits. The experience treatments evaluate how a bee's early experience collecting pollen from intact flowers affects its subsequent response to floral parts and their signal components. Details of system and protocol follow.

Bees

Across all experiments we used 141 workers from seven commercially obtained (Koppert Biological Systems, Howell, MI, U.S.A.) colonies of the bumblebee *B. impatiens* Cresson. We used approximately equal numbers of bees from each colony and in each treatment. To maintain colonies, colonies had access to ad libitum 2 M sucrose solution and pulverized honey bee-collected pollen (Koppert Biological Systems) within plywood test arenas (L×W×H: 82 × 60 × 60 cm and 82 × 60 × 30 cm). Sucrose solution was dispensed via braided cotton wicks that extended into vials. Pollen

was dispensed via custom-made feeders (Russell & Papaj, 2016). The test arenas had clear acrylic ceilings and were lit from above by 40 W and 60 Hz fluorescent lights (Lithonia Lighting, Conyers, GA, U.S.A.) set to a 14:10 h light:dark cycle.

Plants and Flowers

In behavioural trials we used freshly clipped flowers from eight *S. houstonii* Martyn (synonym: *Solanum tridynamum*) plants raised under natural light in a university greenhouse with halogen lights used to extend day length to a 14:10 h cycle. Plants were fertilized weekly (Miracle Gro, NPK = 15-30-15). This species offers only pollen rewards (flowers are nectarless) via tubelike poricidal anthers. Bees extract the pollen by vibrating the anthers, a process termed floral sonication (Buchmann & Cane, 1989). A given trial used an approximately equal number of flowers from each plant. We used 3778 flowers in experiments.

General Experimental Protocol

All trials of each of the three behavioural experiments took place in a test arena (L×W×H: 82 × 60 × 60 cm) painted grey on floor and sides. In experiments, freshly clipped flowers were displayed horizontally (their natural orientation) on custom-built water tubes (see Russell & Golden, 2016) to prevent desiccation. The water tubes were Velcro-mounted on the test arena wall, facing the test arena's nest entrance. Flowers were arranged on the wall in a 3 × 3 Cartesian grid with each water tube spaced 7 cm apart in the horizontal and vertical axes of the grid.

Flowers used in each trial were made unrewarding by gluing the tip of each poricidal anther shut (Elmer's Glue All, Elmer's Products, Atlanta, GA, U.S.A.). Gluing prevented the release of pollen. If bees broke open the anthers and extracted pollen, as they occasionally did, we discarded all visits post-pollen release from analysis. Fresh flowers were used at the start of every trial and for each bee. Flowers were never reused across trials. We systematically alternated treatments that belonged to a given experiment (except for experiment 1; colonies were still equally represented across treatments) in time to control for effects of day and time on behaviour.

To initiate a trial, flowers were first set up in the test arena. We then allowed one to four flower-naïve workers into the arena simultaneously. When one bee landed on a flower (the test bee), we immediately removed the other bees from the arena before they could land on flowers, by catching them in vials and returning them to the colony. While these bees were being captured, the test bee continued to visit flowers and did not exhibit signs of being disturbed by our activity, such as aggressive behaviour or attempts to escape from the arena.

For each trial we recorded three behaviours made by bees visiting flowers: approaches, landings without sonication and landings with sonication ('buzzes' or 'buzzing'). An approach was defined as the bee greatly reducing its velocity while facing the flower, while within 3 cm of the flower (often bees would touch the corolla or anthers with their antennae during approaches). A landing was defined as the bee touching the flower with at least three of its legs simultaneously. All landings were preceded by an approach, although not all approaches were followed by landing. Buzzes, which indicated an attempt at extracting pollen, were identified by their distinctive sound and occurred only after a bee had landed (for extended description, see Russell & Leonard, 2016). Additionally, we recorded to which floral tissue (anther, corolla or surrogate anther) buzzes were delivered, as defined by the placement of the clamped mandibles. To facilitate recording of behaviour, video for all trials was captured at 30 frames/s 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, Fort Worth, TX, U.S.A.) attached to the centre of floral arrays. A Zoom H2 Handy Recorder (ZOOM Corporation, Tokyo, Japan) was used to amplify and verify buzzes in ongoing trials.

During each test trial we counted the flowers on which bees landed in each array across all experiments: each bee visited most flowers in its array at least once. Sometimes a bee visited the same flower more than once in a row, which typically involved the bee landing, hovering while within 3 cm of the same flower, and then relanding on that flower. To be conservative, we discarded these repeat visits (across all treatments, an average of 8% of visits) for all analyses, reasoning that the bee may not have had the opportunity to actively assess the other flowers. We terminated the trial after the bee made 60 approaches in an array or, occasionally, if the bee did not make another approach for a period of 5 min, whichever came first. Most bees made the maximum number of allowed approaches and all but four bees (see individual experiments for details) were included in analyses. A bee was tested only once, after which the bee was euthanized to avoid any transfer of floral cues and *Solanum* pollen to the colony.

Description of Experiments

In the first experiment we sought to determine how the parts of the whole flower, corolla only and anthers only, mediated foraging behaviour of naïve and experienced bees. In the second experiment we investigated the contribution of corolla visual and olfactory cues to the pollen foraging behaviour of naïve and experienced bees. In the third experiment we tested whether anther and pollen chemical cues mediated foraging behaviour by naïve and experienced bees.

In each of these experiments, flower-naïve bees were allocated to either of two types of treatments. In no-experience treatments we assayed flower-naïve behaviour: each bee in these treatments was presented with an array of unrewarding flowers (see individual experiments for details on the types of flowers in arrays). In experiment 1 and 2, the three flower types were assigned to positions in the array, such that all position–flower combinations were equally represented across all trials and no single type of flower appeared more than once in a row or column within a given array. In experiment 3, the two flower types were alternated by position. In experience treatments we assayed the short-term effects of experience on preference, by first training and then testing a separate group of bees. During training, flower-naïve bees were individually presented with three rewarding *S. houstonii* flowers and allowed three landings with sonication, whereupon the rewarding flowers were removed from the arena. We confirmed visually that sonicating bees packed pollen into their corbiculae (pollen baskets). A divider was then lowered and the bee allowed access to the same type of fresh unrewarding array as used in the corresponding no-experience treatment.

Experiment 1: Signalling function of corolla and anther

To assess the function of corolla and anther parts, three types of flowers were represented in each array for a total of nine flowers (Fig 2a–c). The first type consisted of a flower that had its anthers excised where the filament joined with the corolla (the ‘Corolla’ flower type; Fig 2b). Flowers of this type had glue applied at the point where the anther filaments had been severed, as a control for the scent of glue on the other flowers. The second consisted of a flower from which we removed most of the corolla, leaving a circle of tissue to which the stamen filaments were joined (the ‘Anther’

flower type; Fig 2c). The third consisted of a flower from which we removed most of the corolla, as in the second type, and then hot-glued this into the centre of a flower that had its anthers excised to control for cutting the floral tissue (the ‘Sham’ flower type; Fig 2a). We did not observe any wilting or browning of flowers. Control assays comparing sham controls and intact flowers confirmed that cutting and gluing the tissue in this way did not affect bee behaviour (see data in Russell et al., 2016).

This experiment used 53 bees from five colonies. We excluded from analysis a single bee that broke the anthers and obtained pollen on its first landing.

Experiment 2: Importance of corolla visual and olfactory cues

To assess the function of corolla visual and olfactory cues we used a protocol modelled after work on hawkmoths by Raguso and Willis (2002, 2005) and on flies by Policha et al. (2016). Three types of flowers were represented in each array for a total of nine flowers (Fig 2d–f). All of them presented intact anthers, but varied in features of the corolla. The first was a Sham flower (described previously; Fig 2d). The second was a flower whose anthers were removed and the corolla sealed within a transparent oven bag to mask corolla odour, leaving corolla visual cues intact (hereafter, a ‘Bag’ flower; Fig 2e; Reynolds, Inc., Richmond, VA, U.S.A.; see Raguso & Willis, 2005). The third was a flower whose anthers were removed and the corolla concealed under four layers of cheesecloth to mask corolla visual cues, leaving corolla olfactory cues intact (hereafter, a ‘Cloth’ flower; Fig 2f; Rit, Inc., Indianapolis, IN, U.S.A.; see Raguso & Willis, 2005). A clear circular piece of acrylic was added to the back of each experimental flower to protect it from being deformed by the cheesecloth or oven bag. To complete the assembly of Bag and Cloth flowers, we hot-glued anthers to the outside of the bag or cheesecloth-enclosed flower, where the centre of the flower would be (Fig 2e and f).

We tested for potential bias in bee responses to cheesecloth and oven bags by allowing bees to visit a nine-flower array composed of Sham flowers set against cheesecloth, an oven bag or no supplementary background (see Appendix, Fig A1). We confirmed that these backgrounds did not affect bee behaviour (Appendix, Fig A2). Furthermore, we examined the spectra of flowers and of the grey test arena background on which flowers were mounted, and confirmed that the oven bag did not noticeably alter the visual attributes of the corolla and that the cheesecloth was a reasonable match for the background of the test arena; likewise, differences in *B. impatiens* perceptual colour space were minimal (Appendix, Fig A3).

This experiment used 38 bees from two colonies. We discarded from the sample a single bee that broke the anthers and obtained pollen on its first landing.

Experiment 3: Importance of anther versus pollen chemistry

To assess the function of anther and pollen chemistry we used flowers that had substitute foam anthers, to which we added extracts made of *S. houstonii* whole anthers or pollen. We made surrogate flowers by cutting off and discarding the stamens from the corollas of *S. houstonii* flowers. Pure pentane or a pentane extract of *S. houstonii* whole anthers or pollen was applied to surrogate anthers made from Yellow Fibrecraft Foam (Jo-Ann Stores, LLC, Hudson, OH, U.S.A.), cut into cuboids ($L \times W \times H$: $1.4 \times 0.2 \times 0.2$ cm). These surrogate anthers were hot-glued to the corollas of *S. houstonii*, resulting in a surrogate flower (Fig 2g). By retaining the real corollas we were able to strictly assess how the anther and pollen extracts affected foraging behaviour. See Appendix for detailed methods on extract and surrogate flower preparation.

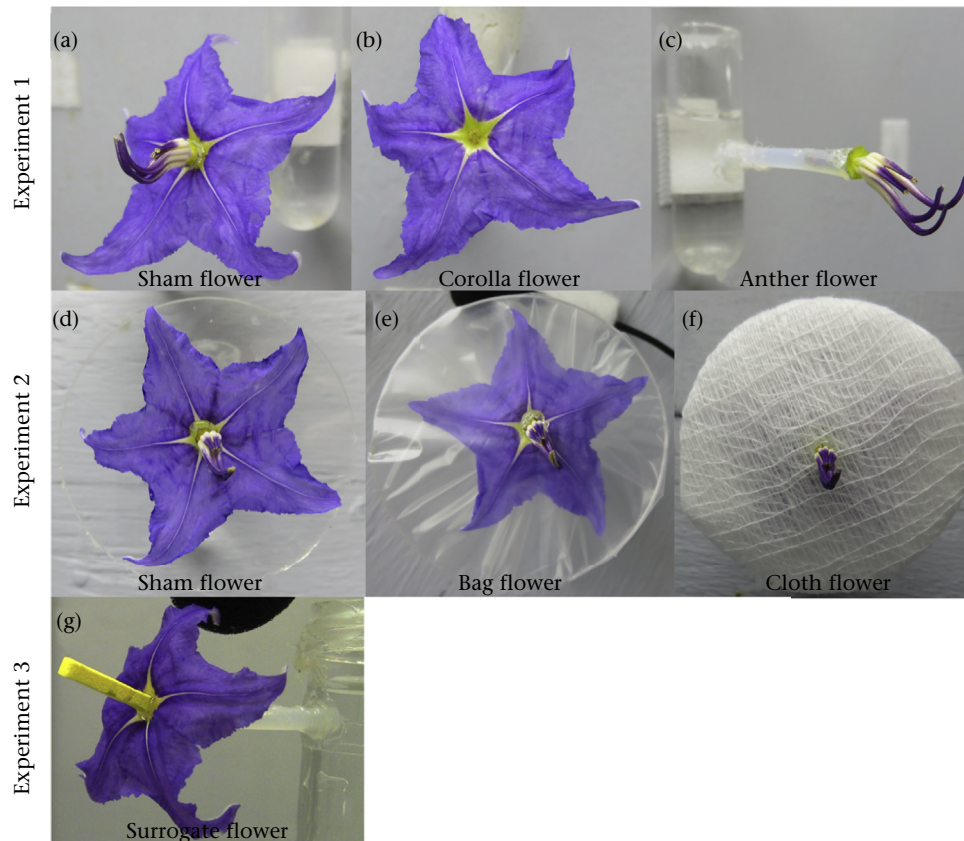


Figure 2. Various flower types made from *Solanum houstonii* used in the study. Experiment 1: (a) anthers glued to corolla (Sham flower); (b) corolla with anthers excised (Corolla flower); (c) anthers with corolla excised (Anther flower). Experiment 2: (d) anthers glued to corolla (Sham flower); (e) corolla sealed within oven bag and anthers glued to the outside (Bag flower); (f) corolla concealed under cheesecloth and anthers glued to the outside (Cloth flower). Experiment 3: (g) surrogate foam anther glued to corolla (surrogate flower). Flowers in (d)–(f) backed by acrylic disk.

The experiment used two no-experience treatments and one experience treatment. Surrogate flowers were arranged in a 3×3 grid without a central flower (eight total flowers). Extracts in one no-experience treatment were made from *S. houstonii* pollen alone and from whole *S. houstonii* anthers in the other no-experience treatment and in the experience treatment. After completion of a trial, each bee in the experience treatment was labelled with individually numbered plastic coloured tags (The Bee Works, Oro-Medonte, ON, Canada) attached by superglue to the dorsum of the thorax and returned to the colony box so that we could assay the long-term effects of experience (Appendix, Fig A4).

This experiment used 43 bees from four colonies. We removed two bees from the study (due to exceptionally low visit numbers): one that completed only three landings with sonication, and another that made only eight approaches.

Data Analyses

All data were analysed using R v3.2.0 (R Core Development Team, 2014). For experiment 1 and 2 we used a hierarchical Bayesian model (BayesPref package) designed for multinomial count data to analyse differences in preference across the three flower types (a detailed description of this analysis and its advantages can be found in Fordyce, Gompert, Forister, & Nice, 2011; Forister & Scholl, 2012; Gompert & Fordyce, 2012). MCMC runs were conducted for 40 000 generations with the first 10 000 generations discarded as burn-in for all analyses. Using the 'plot' diagnostic tool, MCMC samples were examined to confirm even sampling of the posteriors.

We utilized pairwise comparisons of posterior probabilities (i.e. 'PP') to identify significant differences among estimates of preference for each of the three flower types (BayesPref package). When preference for a particular flower type is greater than preference for another flower type – or when preference for a particular flower type in one treatment is greater than preference for that flower type in another treatment – in more than 95% of the sampled MCMC steps, preference estimates are considered to be significantly different (Fordyce et al., 2011). Posterior probabilities can be interpreted similarly to $P - \alpha$ (where $\alpha = 0.05$) in a frequentist approach. Because pairwise comparisons give values for both choice A over B and choice B over A (values that are complementary: A over B is equal to $1 - (B \text{ over } A)$), we report only the smaller value. We use a Bayesian approach (rather than MANOVAs or GLMERs, for instance), because when examining pairwise differences among three or more categories it is the only statistical approach that to our knowledge does not suffer from inflated type I/II error rates when analysing differences between categories that (1) are not independent, (2) lack moderate correlation between dependent variables, (3) have outliers and (4) do not have homogeneity of variances.

Variables analysed in experiment 3 (and for select results in experiment 1) were a composite of each bee's responses (specifically, proportion variables). We analysed differences across flower types in the proportion of approaches and landings, and in the proportion of approaches that resulted in landing (experiment 1) or in sonication. We used paired or two-sample t tests if assumptions of normality and equal variance were met (using Shapiro–Wilk and F tests, respectively, in the 'mgcv' package: Wood, 2015) or,

otherwise, Wilcoxon signed-ranks tests. We also analysed whether naïve bees on their first landing choice had preferences for one flower type over another, using chi-square tests via the 'chisq.test()' function in R.

RESULTS

Experiment 1: Bees Use Corolla Cues to Approach and Land but Use Anther Cues More with Experience

Naïve bumblebees (*B. impatiens*) approached *S. houstonii* flowers that had an intact corolla significantly more frequently than flowers without an intact corolla (Bayesian analysis: Sham versus Anther: $PP < 0.0001$; Corolla versus Anther: $PP < 0.0001$; Fig 3a, Appendix, Table A1). Additionally, there was a marginally nonsignificant tendency for naïve bees to approach flowers that had an intact corolla and anthers more frequently than flowers that lacked anthers (Bayesian analysis: Sham versus Corolla: $PP = 0.053$; Fig 3a, Appendix, Table A1).

Bumblebees that had prior experience collecting pollen from *S. houstonii* likewise approached flowers with corollas more frequently than flowers without corollas (Bayesian analysis: Sham versus Anther: $PP < 0.0001$; Corolla versus Anther: $PP < 0.0001$; Fig 3a, Appendix, Table A1). Experienced bees also approached flowers with intact corollas and anthers more frequently than flowers that lacked anthers to a small but significant extent (Bayesian analysis: Sham versus Corolla: $PP = 0.041$; Fig 3a, Appendix, Table A1).

Experienced bees made a much greater proportion of approaches to these two types over flowers that had only anthers (Bayesian analysis: Sham versus Anther: $PP < 0.0001$; Corolla versus Anther: $PP < 0.0001$; Fig 3a, Appendix, Table A1). Comparing the responses of naïve to experienced bumblebees, experienced bees more frequently approached flowers that had only anthers to a numerically small but nevertheless highly significant degree (Bayesian analysis: naïve versus experienced: Sham: $PP = 0.223$; Corolla: $PP = 0.248$; Anther: $PP < 0.007$; Fig 3a, Appendix, Table A1).

Results for landings were similar in some respects to results for approaches, but with important differences. As with approaches, naïve bees made a greater proportion of their landings on flowers with intact corollas and anthers than on flowers that lacked anthers; however, in this case, the difference was numerically large and highly significant (Bayesian analysis: Sham versus Corolla: $PP < 0.0001$; Fig 3b, Appendix, Table A1). As with approaches, naïve bees made proportionately more landings on flowers with corollas than on flowers that had only anthers, differences that were again highly significant (Bayesian analysis: Sham versus Anther: $PP < 0.0001$; Corolla versus Anther: $PP < 0.0001$; Fig 3b, Appendix, Table A1).

As with approach responses, prior experience collecting pollen from *S. houstonii* enhanced bees' landing responses to *S. houstonii* anthers (Fig 3b), but the effects were more varied and more pronounced. Experienced bees made a significantly greater proportion of their landings on flowers with intact corollas and anthers relative to flowers that had either part removed, and landed much more

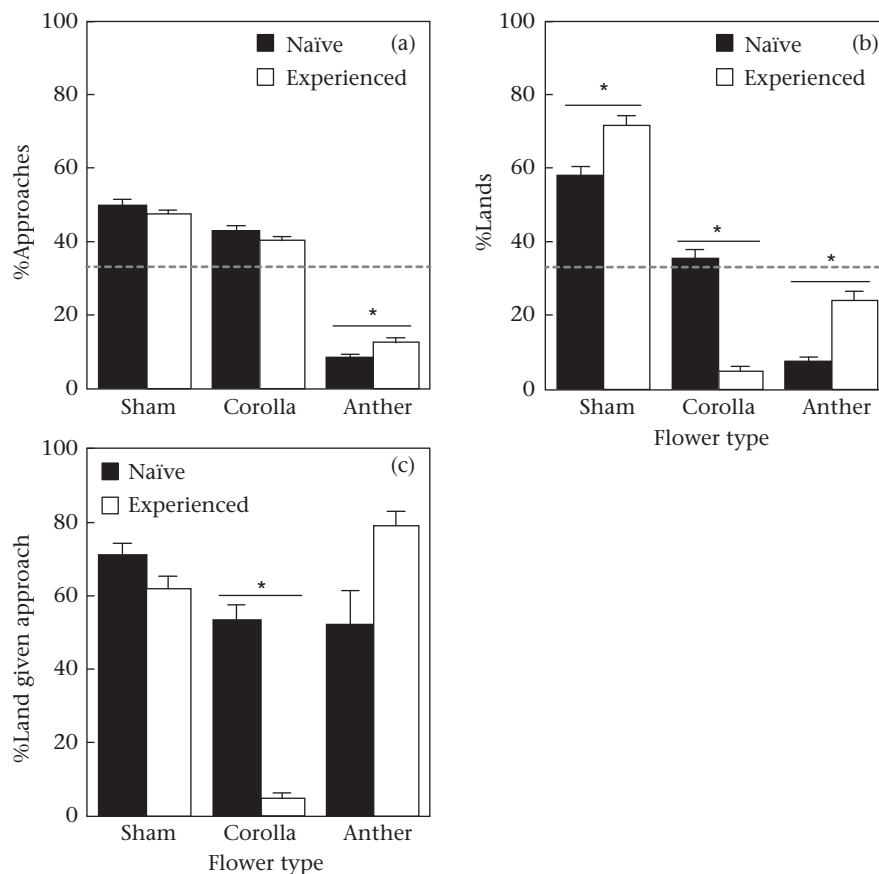


Figure 3. Experiment 1: mean percentage of responses (+SE) by naïve and experienced bumblebees (*Bombus impatiens*) to flowers (*Solanum houstonii*) that had a corolla and anthers (Sham flowers), a corolla only (anthers removed; Corolla flowers), or anthers only (corolla removed; Anther flowers). (a) Approaches and (b) landings for naïve ($N = 31$) and experienced ($N = 21$) bees. Asterisks indicate pairwise differences for naïve versus experienced treatment at posterior probabilities < 0.05 . Dashed line at 33% indicates random expectation for an assay with three choices. (c) Percentage of approaches that resulted in landing for naïve bees ($N = 31$, except for Anther flowers, where $N = 24$, because 7 bees never approached the anthers) and experienced bees ($N = 21$). Asterisks indicate significant differences at $P < 0.05$ (t test or Wilcoxon signed-ranks tests).

frequently on flowers that lacked a corolla over flowers that lacked anthers (Bayesian analysis: Sham versus Corolla: $PP < 0.0001$; Sham versus Anther: $PP < 0.0001$; Corolla versus Anther: $PP < 0.0001$; Fig 3b, Appendix, Table A1). Furthermore, relative to naïve bees, experienced bees landed much more frequently on flowers that had anthers and very rarely landed on flowers without anthers (Bayesian analysis: naïve versus experienced: Sham: $PP < 0.0003$; Corolla: $PP < 0.0001$; Anther: $PP < 0.0001$; Fig 3b, Appendix, Table A1).

Similarly, relative to naïve bees, experienced bees very rarely landed on flowers lacking anthers that they had approached (Wilcoxon two-sample test: naïve versus experienced: $W = 11.5$, $N_{\text{naïve}} = 31$, $N_{\text{experienced}} = 21$, $P < 0.0001$; Fig 3c). A similar effect of experience was not observed on flowers with anthers (Welch two-sample t test: naïve versus experienced: Sham: $t_{47.572} = 1.9014$, $N_{\text{naïve}} = 31$, $N_{\text{experienced}} = 21$, $P = 0.063$; Wilcoxon two-sample test: Anther: $W = 314$, $N_{\text{naïve}} = 24$, $N_{\text{experienced}} = 21$, $P = 0.148$; Fig 3c).

Lastly, the corolla barely elicited pollen extraction behaviour, regardless of experience level: of a total of 909 landings with sonication, just 0.66% involved buzzes delivered to the corolla (rather than to the anthers).

Experiment 2: Advertisement by the Corolla Is Mainly a Function of Visual Corolla Cues

Both naïve and experienced bumblebees (*B. impatiens*) mainly approached *S. houstonii* flowers with intact visual and olfactory corolla cues over flowers that lacked one of these corolla features (Fig 4a). Furthermore, naïve and experienced bees both made significantly fewer approaches to flowers that lacked visual corolla cues than to flowers that had intact corolla visual cues (Bayesian analysis: naïve bees: Sham versus Bag: $PP = 0.044$; Sham versus Cloth: $PP < 0.0001$; Bag versus Cloth: $PP < 0.0001$; experienced bees: Sham versus Bag: $PP = 0.038$; Sham versus Cloth: $PP < 0.0001$; Bag versus Cloth: $PP < 0.0001$; Fig 4a, Appendix, Table A2).

The pattern of landing responses resembled the pattern of approaches, with one exception. As with approaches, bees of both experience levels tended to make a greater proportion of landings on flowers with intact visual and olfactory corolla cues over flowers lacking visual corolla cues or odour cues; however, only naïve bees had a significant preference for flowers with both cues intact over flowers lacking olfactory corolla cues (Bayesian analysis: naïve bees: Sham versus Bag: $PP = 0.0181$; Sham versus Cloth:

$PP < 0.0001$; experienced bees: Sham versus Bag: $PP = 0.059$; Sham versus Cloth: $PP < 0.0001$; Fig 4b, Appendix, Table A2).

For the most part, patterns of approach and landing responses were not influenced by experience collecting pollen from *S. houstonii*, with a single exception: experienced bees approached flowers that lacked visual corolla cues significantly less frequently, relative to naïve bees, although this difference was numerically small (Bayesian analysis: naïve versus experienced: Sham: $PP = 0.225$; Corolla: $PP = 0.345$; Anther: $PP < 0.042$; Fig 4b, Appendix, Table A2).

Experiment 3

Bees use chemical cues associated with the anthers to find the anthers

Both naïve bees and experienced bees approached anther extract-treated surrogate flowers and pentane-treated surrogate flowers equally frequently (paired t tests: naïve bees: $t_{10} = 0.079$, $P < 0.938$; experienced bees: $t_{14} = 0.718$, $P < 0.484$; Welch two-sample t test: naïve versus experienced: $t_{15.39} = -0.263$, $P < 0.796$; Fig 5a).

In contrast, naïve bees landed significantly more frequently on surrogate flowers treated with a pentane extract of live anthers versus on surrogate flowers treated with a pentane-only control (Fig 5a; paired t test: naïve bees, extract versus pentane: $t_{10} = 3.900$, $P < 0.003$, Bonferroni-corrected $\alpha = 0.025$). After bees had experience collecting pollen from *S. houstonii*, the preference to land on anther extract-treated surrogate flowers became significantly more pronounced (Welch two-sample t test: naïve versus experienced: $t_{23.02} = -2.736$, $P < 0.012$, Bonferroni-corrected $\alpha = 0.025$; Fig 5a). These patterns were also mirrored by the first landing choice of bees: more bees landed first on anther extract-treated surrogate flowers than on pentane-only treated surrogate flowers, although this difference was only significant for experienced bees (chi-square test: first landing choice: naïve bees: $\chi^2_1 = 2.273$, $N = 11$ bees, $P = 0.132$; experienced bees: $\chi^2_1 = 5.4$, $N = 15$ bees, $P < 0.021$; percentage of bees that made their first landing on anther extract: naïve bees: 72.7%; experienced bees: 80.0%).

Bees use chemical cues associated with the anthers to extract concealed pollen

Chemical extracts made from *S. houstonii*'s whole anthers, but not of its pollen alone, elicited pollen extraction behaviour by naïve

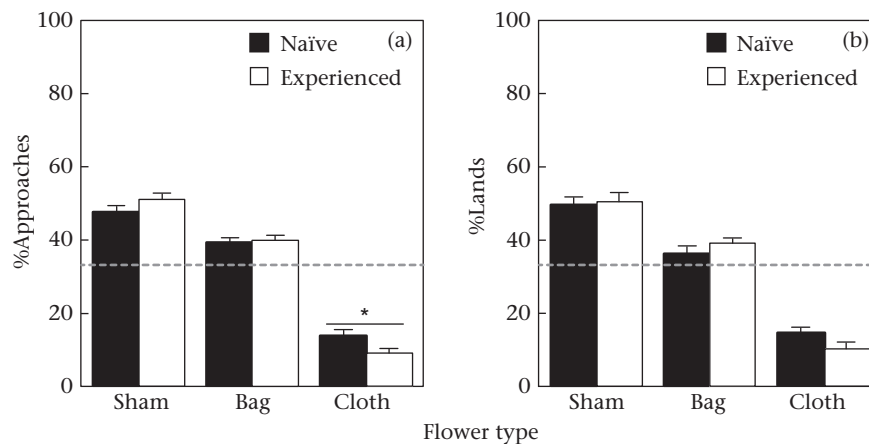


Figure 4. Experiment 2: mean percentage of responses (+SE) by naïve and experienced bumblebees (*Bombus impatiens*) to flowers (*Solanum houstonii*) that had all cues intact (Sham flowers), corolla odour masked (corolla sealed within an oven bag; Bag flowers), or corolla visual cues masked (cheesecloth-wrapped corolla; Cloth flowers). (a) Approaches and (b) landings by naïve ($N = 15$) and experienced ($N = 14$) bees. Asterisk indicates pairwise difference for naïve versus experienced treatment at posterior probabilities < 0.05 . Dashed line at 33% indicates random expectation for an assay with three choices.

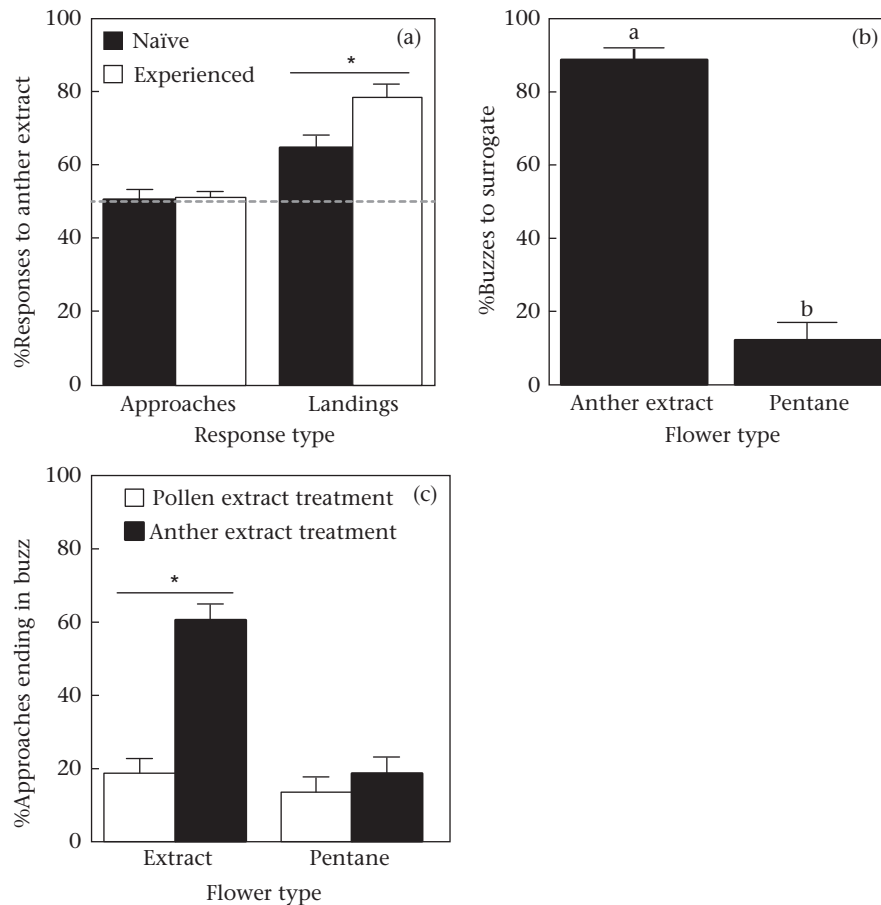


Figure 5. Experiment 3: mean percentage of responses (\pm SE) by *Bombus impatiens* on anther or pollen extract-treated surrogates and on pure pentane-treated surrogates; surrogates made from *Solanum houstonii*. (a) Approaches and landings made by naïve ($N = 11$) and experienced ($N = 15$) bees on anther extract-treated surrogates versus pentane-treated surrogates. Dashed line at 50% indicates random expectation for an assay with two choices. (b) Mean percentage of landings with sonication that specifically involved buzzes to the surrogate anthers (versus the corolla) on anther extract-treated surrogate flowers versus pentane-treated surrogate flowers ($N = 8$ naïve bees, data from (a)). (c) Mean percentage of approaches (\pm SE) that ended in sonication on surrogates treated with anther extract versus on surrogates treated with pentane ($N = 11$ naïve bees for anther treatment (data from (a)), $N = 15$ for pollen extract treatment). Asterisks and different letters above bars within a panel indicate significant differences at $P < 0.05$ (t tests or Wilcoxon signed-ranks test).

bumblebees (Fig 5b; Welch two-sample t test: anther extract assay versus pollen extract: $t_{21,40} = 6.70$, $P < 0.0001$). Additionally, there was no significant difference in the proportion of approaches that ended in sonication for naïve bees visiting surrogate flowers treated with a pollen extract versus surrogate flowers treated with a pentane-only control (paired t test: pollen extract versus pentane: $t_{14} = 1.94$, $P < 0.073$; Fig 5c). On visits where bees buzzed anther extract-treated flowers, buzzes were overwhelmingly performed on the anther extract-treated surrogate anthers; when bees buzzed pentane-treated surrogate flowers they mainly buzzed where the excised filaments had been (Wilcoxon signed-ranks test: anther extract versus pentane, percentage of lands with sonication to the surrogate anther: $W = 80$, $N = 8$, $P < 0.0006$; Fig 5c).

DISCUSSION

Complex signals are typically transmitted by a variety of morphologically distinct parts (Girard et al., 2011; Leonard et al., 2012; Preininger, Boeckle, Szatecsny, & Hödl, 2013). The cues that make up complex signals often vary among morphological parts (e.g. Girard et al., 2011; Leonard et al., 2012). Such variation affects signal content (e.g. Dobson, Danielson, & van Wesep, 1999; Girard et al., 2011; Leonard et al., 2012; Raguso & Willis, 2002), and thus different physical parts might contribute to different

components of the complex signal. We found that signal components of the complex floral signal differ between two such morphologically distinct parts: the corolla and the anthers. The corolla initially serves two functions, to elicit approach and then to elicit landing. The anther also serves two functions from the start; to elicit landing and then to elicit the extraction of pollen (via floral sonication). The floral parts also interact. Initially, both corolla and anther elicit landing given approach, and their stimulatory effects add together to an extent. Our findings furthermore suggest the relationship between specific floral parts and specific signal components evolves to improve the overall functionality of the complex signal. In our study, the corolla initially enhanced receipt of pollen (the male plant gamete) from the bee by eliciting attraction and landing (critical for pollen transfer) regardless of the presence of a reward. The anthers enhanced transfer of pollen to bees by arresting them after they had landed, and eliciting extraction of pollen from these pollen-bearing parts.

The relationship between floral part and signal component function in a complex signal is not static but changes rapidly with experience. Corolla and anther both function initially to elicit landing. However, as the bee gains experience, the anther alone elicits landing. Accordingly, experience affects how the floral parts interact, with corolla and anthers no longer acting together to elicit landing. Because anther cues are directly relevant to pollen

collection, by learning to rely more completely on anther cues bees might forage more effectively (see also Burger, Dötterl, & Ayasse, 2010; Dobson & Bergström, 1999; Milet-Pinheiro, Ayasse, Schlindwein, Dobson, & Dötterl, 2012). Responses to floral signals that are altered with experience frequently benefit plant and pollinator (Lewis, 1993; Raine & Chittka, 2012). While Russell et al. (2016) showed that experience has only a small influence on the speed with which bees access the concealed pollen reward, learning of anther cues might provide other benefits. For instance, bees might accept the flowers of species they are familiar with more quickly or with greater accuracy (e.g. Kulahci, Dornhaus, & Papaj, 2008; Ings & Chittka, 2008; Russell et al., 2016), thereby improving both foraging and pollination.

Furthermore, pollinators might forage more efficiently and reduce risk of predation if they were able to ascertain reward status prior to landing on the flower (Hansen, Van der Niet, & Johnson, 2012; Howell & Alarcón, 2007; Leonard & Papaj, 2011). Our results however suggest that generalist bumblebees are unable to use cues directly associated with concealed pollen status to determine whether to attempt pollen extraction from plant species with poricidal floral morphology (see also Buchmann & Cane, 1989; Burkart, Schlindwein, & Lunau, 2013; Connolly & Anderson, 2003). Instead, cues produced by the anthers alone elicit pollen extraction (floral sonication). Thus bees are encouraged to attempt to extract the pollen reward even from flowers that are depleted of pollen, probably facilitating the continued transfer of pollen to formerly rewarding flowers. Indeed, this behavioural pattern may have facilitated the relatively frequent evolution of so-called cryptic dioecy among plant species with poricidal floral morphology, a plant mating system wherein flowers of both sexes have anthers, but anthers of only one sex produce pollen (Lunau, 2007; Penny, 2014; Walker-Larsen & Harder, 2000; Wang & Hu, 2011 and references within).

Our study of floral signal components suggests that as with behaviours such as mate and host selection, flower visiting is likely a catenary process (Kennedy, 1965; Raguso & Willis, 2002), involving a chain of linked behavioural responses to floral stimuli. At any given step in the sequence, perception of floral stimuli results in the pollinator encountering stimuli in the following step, and so on until pollen is transferred to the pollinator. Bees are drawn to the conspicuous corolla from a distance; when in range of anther cues, bees land, whereupon closer range anther cues elicit pollen transfer (via floral sonication). With floral experience, bees' dependence on corolla cues is lessened but not eliminated. This catenary process appears to be facilitated by the relationship between the floral part and its cues. Corolla conspicuousness in our study is largely a function of the visual modality, while short-range chemical cues appear to be important for the signalling function of the relatively smaller anther (see also Orbán & Plowright, 2014 and references within). In fact, anther chemical cues do not act from a distance to elicit approaches, and olfactory corolla cues likewise elicit few approaches by bees. Why do bees not rely more on corolla olfactory cues? Work in progress suggests that corolla olfactory cues might be largely absent: the anthers of *S. houstonii* appear to emit most of the flower's olfactory compounds and in much higher concentrations (Russell, Kessler, Buchmann, & Papaj, n.d.), a pattern that is similarly found in other flowering plant species (e.g. Burdon et al., 2015; Dobson et al., 1996). Such a pattern may have evolved, if, for instance corolla olfactory cues interfered with anther signal function.

How might the composition of complex signals produced by other flowering plant species vary among floral parts? Plant species spread across at least 17 plant families (De Luca & Vallejo-Marín, 2013; Faegri, 1986; Russell et al., 2016) exhibit floral morphology convergent with *S. houstonii*: the solanoid floral form. Solanoid flowers are typically nectarless and possess poricidal floral morphology, and are thus typically pollinated by pollen-foraging

bees capable of using floral sonication (Buchmann, 1983; De Luca & Vallejo-Marín, 2013). Accordingly, comparable selective pressures by pollinators may have resulted in such plant species dividing signal components among their anthers and corolla as we observed here in *S. houstonii*.

Yet many flowering plant species possess different floral morphology and offer different combinations of floral rewards (see Kevan & Baker, 1983; Simpson & Neff, 1981; Westerkamp, 1999). For instance, pollen signals may be predominant in plant species that offer pollen on the surfaces of their anthers (Cresswell & Robertson, 1994; Dobson et al., 1999; Dötterl & Vereecken, 2010; Goulson, Chapman, & Hughes, 2001), suggesting that poricidal anthers, which conceal pollen cues, assumed the signalling function of pollen to some extent. Furthermore, it is plausible that selection may favour reduced anther and pollen signalling function in plants offering nectar rewards (but see Ashman et al., 2005; Lunau, 1992; Lunau, Unseld, & Wolter, 2009; Pohl, Watolla, & Lunau, 2008). Such a pattern might serve to restrict excessive pollen removal by pollinators, which can be a considerable selective pressure (Castellanos, Wilson, Keller, Wolfe, & Thomson, 2006; Hargreaves, Harder & Johnson, 2009). Consistent with this, signals associated with nectar appear most important in eliciting landing, feeding and presumably pollen transfer by nectar-foraging pollinators (Howell & Alarcón, 2007; Raguso & Willis, 2005; Raguso, 2004).

In closing, the study of complex signals in animals can be challenging because both signaller and receiver behave. Likewise, it is often difficult to isolate and manipulate their signal components and the morphological parts that convey them. By comparison, flowers are more static and components of floral displays can be readily mimicked for instance via artificial surrogates and manipulated using selective breeding (e.g. Bradshaw & Schemske, 2003; Muth et al., 2016). By cutting, gluing together and concealing parts of live flowers (e.g. Johnson, Delph & Elderkin, 1995; Waser & Price, 1985; Raguso & Willis, 2005), we find that signal components may be divided to some extent among particular flower parts. Furthermore, we find that the particular relationship between the signal component and its morphological part appears to enhance the effectiveness of the complex signal. Future work will be required to understand how manipulating the degree to which signal components are divided among parts affects the function of the complex signal. We suggest that the study of complex signalling evolution would benefit from an increased understanding of how signal components relate to the physical parts that transmit them, not just in flowering plant species, but in other organisms such as spiders, fish, frogs, lizards and birds that use complex signals.

Acknowledgments

We are grateful to Steve Buchmann, Judie Bronstein and Annie Leonard for helpful comments, to Abreeza Zegeer for greenhouse care and to Sarah White and Eleni Moschonas for assistance in running experimental trials. This work was supported by the Graduate & Professional Student Council of the University of Arizona and the National Science Foundation (IOS-1257762).

Supplementary material

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.anbehav.2017.11.021>.

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Appendix

Preparation of Pentane Pollen Extracts

Each pollen extract was made from an equivalent of 60 flowers' pollen, as determined by measuring the average amount of pollen in *S. houstonii* flowers (2.01 mg of pollen/flower when the study was conducted). We used pollen frozen at -18°C that had been removed from the flowers of three *S. houstonii* plants on 4 December and 8 December 2013 using an ultrasonicator (Virtis Virsonic 100, Boston Laboratory Equipment, Woburn, MA, U.S.A.). Using a Sartorius Analytic Balance (Data Weighing Systems, Elk Grove Village, IL, U.S.A.) we weighed out 120 mg of pollen and placed pollen into sterile 2.0 ml amber autosampler vials (Thermo Fisher Scientific, Waltham, MA) with 1.2 ml of pentane. After 5 min, extracts were transferred to fresh vials; the pollen and ~0.2 ml of pentane was left in the original vial. Pollen extracts thus contained an equivalent of 60 flowers' pollen in 1.0 ml of solvent. Prepared

extracts were stored at -18°C . See Russell, Buchmann, and Papaj (2017) for detailed methods on surrogate flower and pentane anther extract preparation.

Reflectance and Irradiance Spectra and Bee Colour Space

To determine how bees visually perceived each flower type in experiment 2, we characterized the colour of Sham, Bag and Cloth corollas, and the background against which the flowers were presented in experiments, using reflectance and irradiance spectra (Fig A3). Each reflectance spectrum consists of the mean of five measurements. Each measurement was taken from a flower (or part of the test arena). Reflectance spectra for all samples were measured using an UV–VIS spectrophotometer (Ocean Optics USB2000; Ocean Optics, Dunedin, FL, U.S.A.) with tungsten-deuterium light source (Ocean Optics DH2000) and a fluoropolymer white standard (USRS-99-010 Spectralon; Labsphere, North Sutton, NH, U.S.A.). An RPH reflectance probe (Ocean Optics) was held at constant height and angle above the samples using a holder that shielded the probe from extraneous light. Reflectance measurements were taken using a 5 ms integration time in the same session. Irradiance within the testing arena was measured at the centre of the foraging array using a P600 UV/VIS optical fibre (Ocean Optics), a CC-3-UV-T cosine-corrected (180 degrees) irradiance probe (Ocean Optics) and a tungsten-deuterium calibration light source (DH2000, Ocean Optics). Irradiance measurements were taken using a 50 ms integration time.

To characterize what bees perceived, we used our reflectance and irradiance measurements to plot colour morphs within a colour space for *B. impatiens*. The colour space diagram (i.e. colour hexagon) and table were made in accordance with Chittka (1992), using data on receptor spectral sensitivities for *B. impatiens* from Skorupski and Chittka (2010). We used the test arena wall on which the flowers were displayed as the background stimulus for the colour hexagon and the irradiance of the overhead arena lights in calculations of receptor excitation values. Sham and Bag flowers were similar to each other in bee colour space, as were Cloth flowers to the background (Fig A3).

Control for Oven Bag and Cheesecloth Used to Modify Flowers

To assess potential bee bias in responses to the oven bag or cheesecloth, we examined preference in an unrewarding nine-flower array. This experiment used seven bees and two colonies. The array consisted of three types of flowers: Sham flowers set against a cheesecloth (Cloth), an oven bag (Bag) or no supplementary background (Sham). We found no significant differences in the proportion of approaches or landings (differences in pairwise posterior probabilities: approaches: Sham versus Bag: $PP = 0.318$; Sham versus Cloth: $PP = 0.209$; Bag versus Cloth: $PP = 0.386$; landings: Sham versus Bag: $PP = 0.222$; Sham versus Cloth: $PP = 0.281$; Bag versus Cloth: $PP = 0.429$; Fig A2).

Long-term Memory of Anther Chemistry

To assay the long-term effects of experience, tagged bees that had completed the short-term retention treatment in experiment 3 were tested again approximately 24 h later. Twelve of 16 bees were successfully retested. During testing we presented the bee with unrewarding surrogate flowers arranged in a 3×3 grid without a central flower (eight total flowers), with pentane-only control and anther extract-treated flowers alternated by position.

Experienced bees tested 24 h after their first rewarding experience retained their memory of the anther chemistry. Specifically, these experienced bees exhibited a significant preference to land on surrogate flowers treated with the anther extract versus pentane-treated surrogate flowers (paired t test: extract versus

pentane: $t_{11} = 5.319$, $P < 0.0003$; Fig A4). Furthermore, the strength of this preference did not differ significantly from the bees' initial learned preference 24 h prior (Welch two-sample t test: long term versus short term: $t_{21.97} = 0.920$, $P = 0.368$; Fig A4).

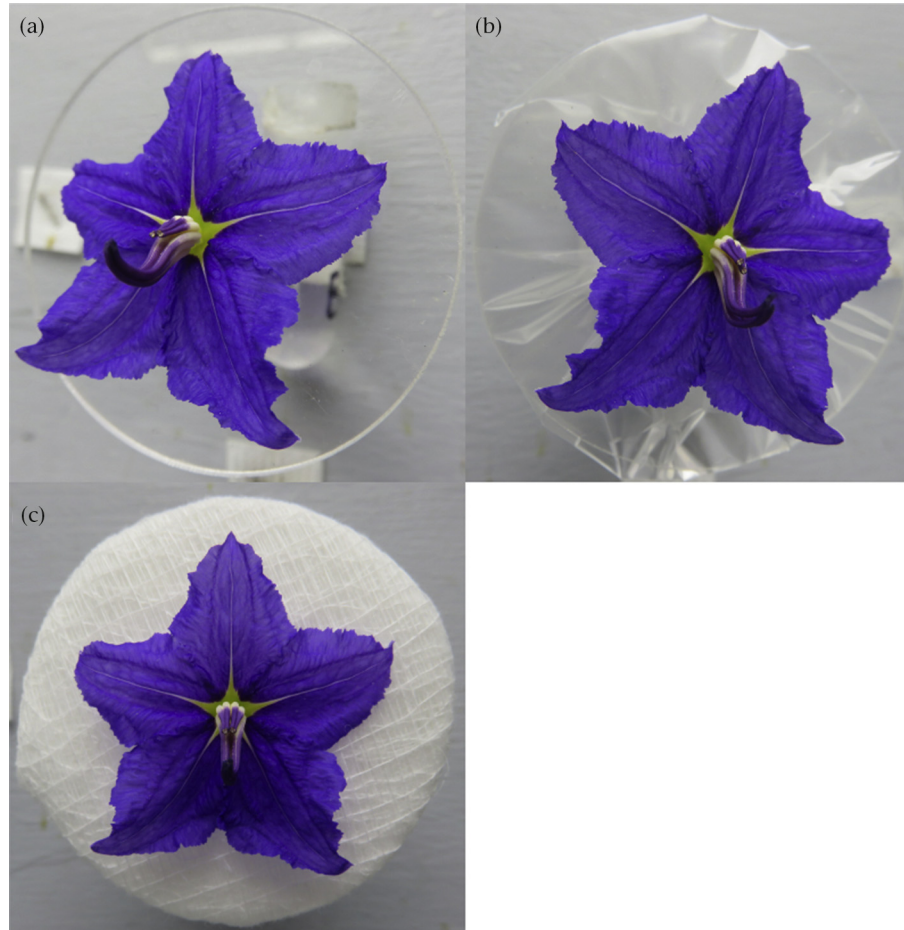


Fig. A1. Sham *Solanum houstonii* flowers set against (a) no background, (b) an oven bag background and (c) a cheesecloth background. Sham flowers were constructed by hot-gluing anthers to corolla, as a control for cutting of floral parts in related experiments.

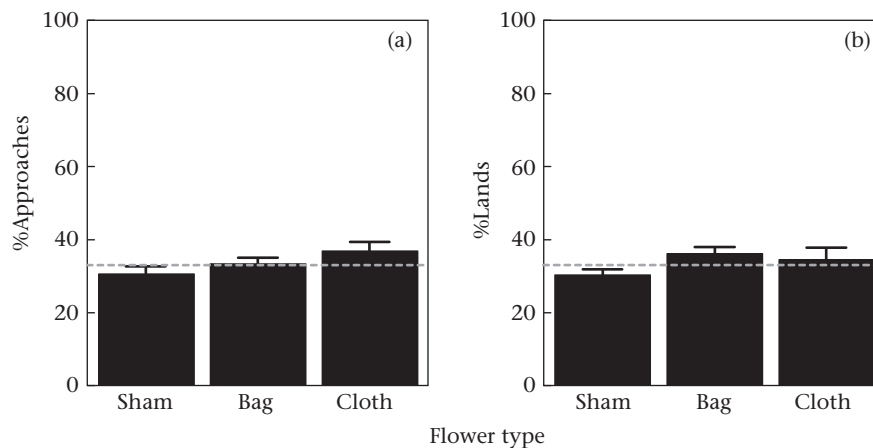


Fig. A2. Mean percentage of responses (\pm SE) by naïve bumblebees (*Bombus impatiens*) to sham flowers (*Solanum houstonii*) set against an oven bag (Bag flowers), a cheesecloth (Cloth flowers) or no background (Sham flowers). (a) Approaches and (b) landings ($N = 7$ bees). Dashed line at 33% indicates random expectation for an assay with three choices.

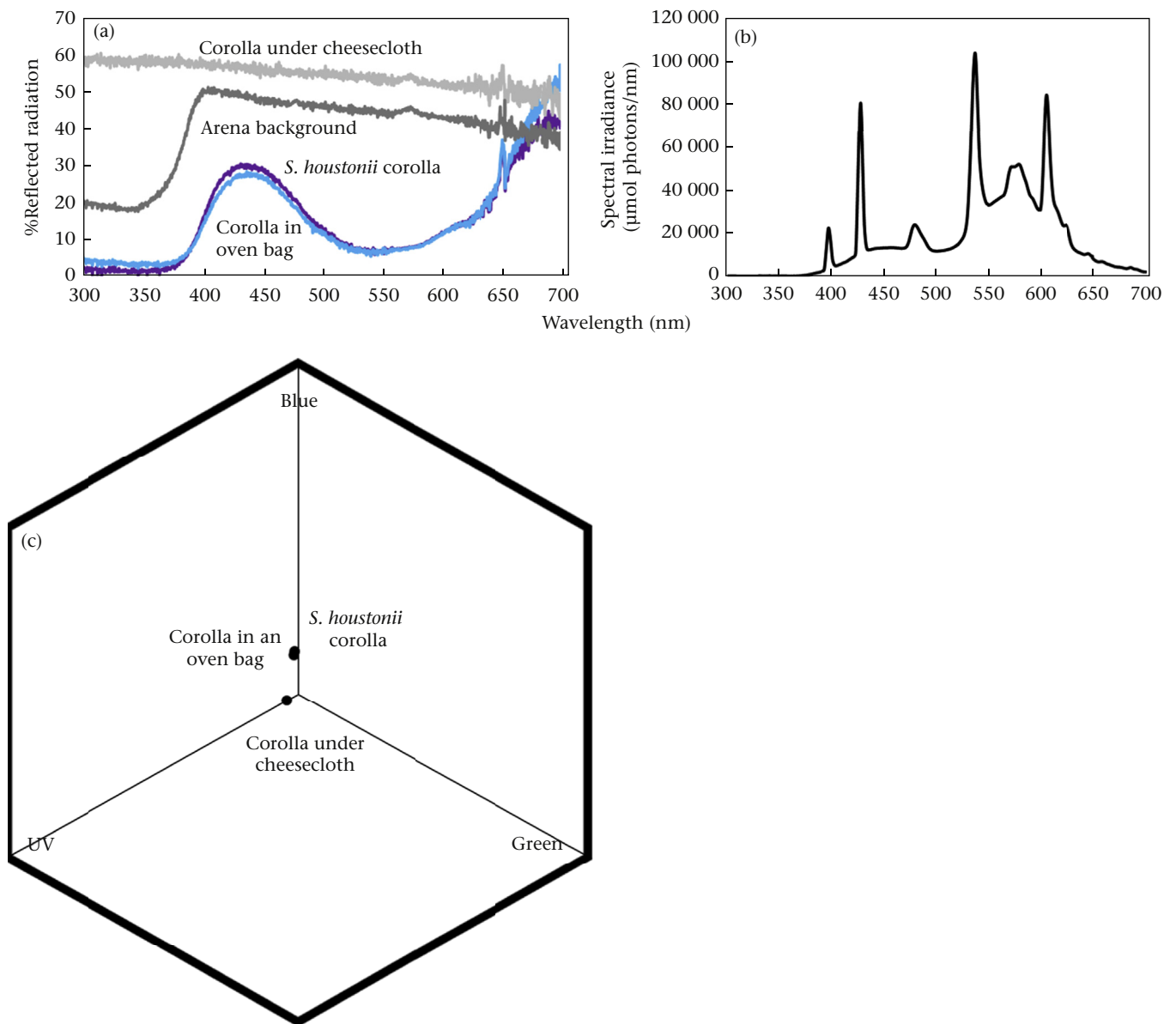


Fig. A3. (a) The reflectance spectra of the test arena background and of the three types of *Solanum houstonii* flowers (Sham, Bag and Cloth flowers) used in experiment 2 (average of the five readings). For spectra taken of flowers, spectra were made from the peripheral tissue and not the yellowish central tissue. (b) The irradiance of the fluorescent lights illuminating the test arena. The reflectance of corollas under cheesecloth was comparable to corollas not covered by anything. Cheesecloth increased reflectance by approximately 10.7% relative to the test arena background at 400–700 nm; below 400 nm the difference became great (up to ca. 40%); however, the fluorescent light sources did not produce light below ~400 nm, so this difference is irrelevant. (c) The loci in *Bombus impatiens* colour space of Sham, Bag and Cloth flowers against the test arena background. Sham and Bag flowers resembled each other, as did Cloth flowers and the background. It is difficult for bumblebees to discriminate targets less than 0.07 colour units apart; the cheesecloth and arena background differ by only 0.044 colour units (Dyer, 2006).

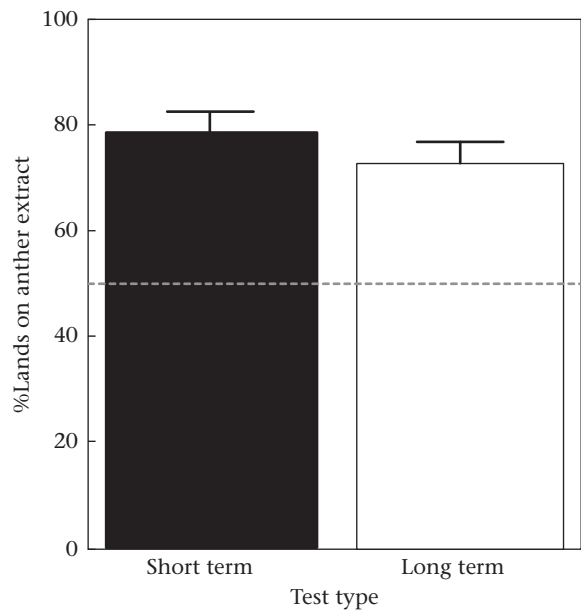


Fig. A4. Mean percentage of landings (+SE) by *Bombus impatiens* on anther extract-treated surrogates (versus pure pentane-treated surrogates); surrogates constructed from *Solanum houstonii* flowers and foam. Data shown for bees' short-term retention tests ($N = 16$ bees) and long-term retention tests ($N = 12$ bees). Dashed line at 50% indicates random expectation for an assay with two choices.

Table A1
Experiment 1, differences in posterior probabilities via a Bayesian analysis

	Naïve		Experienced		Naïve vs Experienced	
	Approaches	Landings	Approaches	Landings	Approaches	Landings
Sham vs Corolla	0.053	<0.0001	0.041	<0.0001		
Sham vs Anther	<0.0001	<0.0001	<0.0001	<0.0001		
Corolla vs Anther	<0.0001	<0.0001	<0.0001	<0.0001		
Sham					0.223	<0.0003
Corolla					0.248	<0.0001
Anther					<0.007	<0.0001

Table A2
Experiment 2, differences in posterior probabilities via a Bayesian analysis

	Naïve		Experienced		Naïve vs Experienced	
	Approaches	Landings	Approaches	Landings	Approaches	Landings
Sham vs Bag	0.044	0.018	0.038	0.059		
Sham vs Cloth	<0.0001	<0.0001	<0.0001	<0.0001		
Bag vs Cloth	<0.0001	<0.0001	<0.0001	<0.0001		
Sham					0.225	0.432
Bag					0.345	0.226
Cloth					<0.043	0.094