MECHANISMS OF FLORAL SPECIALIZATION BY POLLEN-FORAGING BUMBLE BEES

by

Avery Leigh Russell

A Dissertation Submitted to the Faculty of the

GRADUATE INTERDISCIPLINARY PROGRAM IN ENTOMOLOGY & INSECT SCIENCE

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2016

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Avery Russell, titled *Mechanisms of Floral Specialization by Pollen-Foraging Bumble Bees* and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

_____ Date: (11/21/2016)
Daniel R. Papaj

_____ Date: (11/21/2016)
Wulfila Gronenberg

_____ Date: (11/21/2016)
Judith L. Bronstein

_____ Date: (11/21/2016)
Stephen L. Buchmann

_____ Date: (11/21/2016)
Anne S. Leonard

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

_____ Date: (12/4/2016)
Dissertation Director: Daniel R. Papaj

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of the requirements for an advanced degree at the University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

SIGNED: _____ Avery L. Russell

ACKNOWLEDGEMENTS

This doctoral dissertation reflects the combined efforts of a great number of intelligent and considerate people. First, I thank my advisor, Dan Papaj, who welcomed me into his lab, introduced me to the incredible world of animal behavior, and gave me a great many opportunities that I will always be grateful for. Dan has been instrumental in teaching me how to think, write, and communicate clearly and I am most appreciative of his continued mentorship and support. Judie Bronstein has been a powerful role model and I thank her for the hours of advice, encouragement, and advice she has given me. I thank Steve Buchmann for his friendship, his encyclopedic knowledge and passion for bee research, and for taking us on many excursions to see what animals actually do outside of the lab. I thank Wulfila Gronenberg for his tremendous hospitality, for grounding my graduate career, for teaching me many valuable skills, and for allowing me to occasionally monopolize his microscopes. I thank Annie Leonard for her knowledge, support, and for reading over countless manuscript drafts. The dissertation committee and the Bronstein, Gronenberg, Leonard, and Papaj labs have been key and tremendous sources of support and friendship.

Many people conducted these experiments and they deserve plentiful praise. I could not have asked for a finer group of research assistants, whose enthusiasm, hard work, compassion, and friendship made mentoring a joy. Of the many students who have worked with me, my thanks especially to (alphabetized) Heather Gillette, Bekah Golden, Tara Hall, Kevin Mauerman, Mary McIntosh, Eleni Moschonas, China Rae Newman, Cynthia Trefois, and Sarah White. I would also like to thank the EEB, CIS, and EIS staff, especially Sky Dominguez, Lauren Harrison, Marilyn Kalthoff, Teresa Kudrna, Rachael Mattull, Kristina Souders, and Pat Waters, for diligently documenting all my weirdly wonderful shipments and for taking care of the huge amount of paperwork and scheduling necessary to keep a dissertation going. This work would likewise have been impossible without the greenhouse assistance of Abreeza Zegeer, who taught me many valuable horticultural tricks. For making me feel at home in EEB, EIS, and Neuroscience, in addition to those mentioned previously I must also thank, in alphabetical order: Yves Carrière, Goggy Davidowitz, Katrina Dlugosch, Kirsten Grabo, Molly Hunter, Wendy Moore, Lynne Oland, Nick Strausfeld, Bruce Tabashnik, Kathleen Walker, and Konrad Zinsmaier.

I also thank the numerous graduate students and postdocs who contributed brains, companionship, coffee, and beer to keep me functional – and for not running away from an unending stream of puns. In alphabetical order, these wonderful people include, but are not limited to Sarah Bengston, Kathryn Busby, Tuan Cao, Bodil Cass, Dan Charbonneau, Carla Essenberg, Garrett Hughes, Eileen Jeffrey, Elinor Lichtenberg, Chan Lin, Brianna McTeague, Chandreyee Mitra, Sarah Morrison, Felicity Muth, Matt Nielsen, Robert Orpet, John Palting, Sarah Richman, Andre Riveros, Pedro Rodrigues, Eric Shuman, Gordon Smith, Andy Stiegler, Corinne Stouthamer, Jessica Vogt, Lisa Wang, Kara Welch, and Gabby Wolff. *And the list goes on...* Lastly, I thank the spiny plants and the stinging bees for cooperating most of the time, and for ibuprofen and Epipens when they did not.

This research was funded by grants from the Center for Insect Science (UA), NSF (Award Nos. IOS-0921280 and IOS-1257762), the Graduate and Professional Student Council (UA), the Entomology and Insect Science Program (UA), and the Department of Entomology (UA).

DEDICATION

To my family, teachers, and mentors. I would especially like to thank John & Yvi Russell, who have always supported me and encouraged a love of the natural world and the humanities; and my partner-in-crime: the intelligent and amazing Sarah Morrison.

TABLE OF CONTENTS

ABSTRACT……8

INTRODUCTION……10

PRESENT STUDY……16

REFERENCES……..20

APPENDIX A: 23

PATTERNS OF POLLEN AND NECTAR FORAGING SPECIALIZATION BY BUMBLEBEES OVER MULTIPLE TIMESCALES USING RFID

APPENDIX B: ……..75

BEES LEARN PREFERENCES FOR PLANT SPECIES THAT OFFER ONLY POLLEN AS A REWARD

APPENDIX C: ……129

SYNERGY BETWEEN VISUAL AND OLFACTORY CUES IN ASSESSMENT OF CONCEALED POLLEN REWARDS BY BUMBLE BEES

APPENDIX D……..178

CONCEALED FLORAL REWARDS AND THE ROLE OF EXPERIENCE IN FLORAL SONICATION BY BEES

APPENDIX E: …….....226

HOW A GENERALIST BEE ACHIEVES HIGH EFFICIENCY OF POLLEN COLLECTION ON DIVERSE FLORAL RESOURCES

ABSTRACT

A fundamental question in biology is how animals efficiently locate and use diverse resources. Pollinators foraging on flowers are one of our most thoroughly studied examples of generalist foraging behavior and cognition. Individual pollinators typically specialize on a subset of flowering species available to them. Specialization by nectar-foraging pollinators is often the consequence of learned or innate preferences for floral display traits such as color, pattern, and scent. Pollinators must also typically learn to extract nectar from each floral type. By specializing, pollinators reduce costs associated with learning and forgetting nectar extraction routines. Specialization also benefits the plant by enhancing conspecific pollen transfer. Yet nectar is not the only floral reward. The pollen of hundreds of thousands of plant species is collected by pollinators such as bees, beetles, and flies. In fact, solitary and social bees must collect both pollen and nectar to survive. However, much of the vast literature on bee foraging behavior concerns the collection of nectar. This research investigated mechanisms by which generalist bumblebees (*Bombus impatiens*) specialize on diverse floral resources. Most foragers in a colony were reward generalists over their lifetime, but specialized daily on either pollen or nectar collection. Lifetime patterns of pollen collection were associated with interindividual differences in sensory morphology. Pollen-foraging bumblebees had weak innate preferences, but learned strong preferences for pollen-only plant species, with preferences mediated primarily by anther properties. The anthers provided indirect cues of concealed pollen, and bees learned to prefer properties of the anthers to select potentially rewarding flowers. While learning was involved in the formation of floral preferences by pollen foragers, pollen extraction behavior relied little on learning.

Specifically, floral sonication, which is used by bees to extract concealed pollen, was modified only modestly with experience. Furthermore, bees foraged efficiently for pollen from diverse floral resources without relying on instrumental (associative) learning. Efficient foraging involved switching between two distinct motor routines: floral sonication and scrabbling. Switching was regulated by two ubiquitous floral cues: chemical anther cues eliciting sonication and mechanical pollen cues suppressing it (and eliciting scrabbling). I discuss how mechanisms of floral specialization by generalist pollen-foraging bees could drive floral trait evolution.

INTRODUCTION

An explanation of the problem and review of the literature

A subject of intense study in biology is the evolution and maintenance of specialization. Specialization, to varying degrees, is found at all levels of biological organization and has been particularly well-studied in the context of plant-pollinator mutualisms (e.g., Chittka et al. 1999; Page et al. 2009). Specialization by pollinators on their floral hosts is thought to drive much of the wonderful diversity of floral form (Chittka et al. 1999; Gegear & Burns 2007; Schiestl & Johnson 2013; Hopkins & Rausher 2014). By specializing on a particular plant type (e.g., a morph or a species), pollinators maximize pollen transfer among individuals of a given type, while simultaneously minimizing pollen transfer between types (Waser 1986; Chittka et al. 1999). Such assortative mating can facilitate the evolution of floral traits that further enhance pollinator specialization and can eventually lead to divergence and speciation in flowering plants (Gegear & Burns 2007; Anderson et al. 2009; Schiestl & Johnson 2013; Hopkins & Rausher 2012). Floral specialization also provides benefits to the pollinator. Pollinators that specialize on a given floral type minimize switching costs and often develop skills to forage more efficiently for nectar on that flower type (Lewis 1993; Chittka et al. 1999). Further, specialization by individual workers in eusocial pollinator species is thought to benefit the entire superorganism and thereby contribute to their ecological success (Dornhaus 2008; Chittka & Muller 2009).

Two mechanisms in particular, innate bias and learning, are thought to drive floral specialization by pollinators. Innate biases can predispose a pollinator to prefer particular

floral traits over others, resulting in specialization by that pollinator on the preferred floral type (Schiestl & Johnson 2013). For instance, a pollinator that has an innate sensory bias for blue coloration will prefer to visit blue flowers over flowers of other colors (e.g., Raine & Chittka 2007). Innate biases may likewise account for patterns of foraging task specialization by eusocial pollinators. Honeybees for instance are thought to specialize on collecting one resource versus another as a consequence of interindividual variation in sensitivity to cues that stimulate collection of a given resource (i.e., the 'response threshold model'; Page et al. 2006, 2009; Riveros & Gronenberg 2010). Interindividual variation in response thresholds is in turn thought to be a consequence of differences in sensory morphology (Riveros & Gronenberg 2010).

Learning also contributes to specialization by pollinators. Learning is frequently associative, involving enhancement of pollinator responses to floral traits paired with a floral reward (Papaj & Lewis 1993). Nectar foraging pollinators can learn floral display traits including floral color, pattern, scent, microtexture, and electrical fields (Giger and Srinivasan 1995; Gumbert 2000; Whitney et al. 2009; Whitney et al. 2009; Clarke et al. 2013; Schiestl & Johnson 2013; Foster et al. 2014). Learning often results in the formation of strong and long-lasting floral preferences by pollinators (see Giurfa 2007). Additionally, extraction of nectar from flowers frequently requires learning (Gegear & Laverty 1995; Laverty 1994). Nectar is frequently concealed within complex flowers, requiring the use of complex motor routines by pollinators to extract that nectar efficiently (Laverty & Plowright 1988; Westerkamp 1999). These nectar extraction routines are frequently learned and must often be relearned when the pollinator switches

to another flower type and back again (Chittka et al. 1999). Costs associated with learning, remembering, and relearning nectar extraction routines are understood to encourage floral specialization by pollinators (Lewis 1993; Gegear & Laverty 1995; Chittka et al. 1999). Accordingly, the evolution of complex floral morphology that requires the use of learned nectar extraction routines may be a strategy by which plants enhance their pollination (Plowright & Laverty 1984; Lewis 1993; Chittka et al. 1999).

While nectar is a common reward, pollinators such as bees, many beetles and flies, and even some butterflies and wasps (Simpson & Neff 1981; Kevan & Baker 1983) collectively collect pollen from hundreds of thousands of plant species. Bees in particular, whether social or solitary, must collect pollen, their primary source of protein (Plowright & Laverty 1984; Nicolson 2011). Bees in fact are considered a model system for the study of pollinator behavior and cognition (Giurfa 2007; Menzel 2012; Leonard & Masek 2014). Yet much of the vast literature on mechanisms of floral specialization by bees relates only to nectar as a reward (Plowright & Laverty 1984). While little research has examined plant adaptations that support the use of pollen as a reward that facilitates pollen transfer (versus the use of pollen by pollinators without actually improving pollination), at a minimum, species that offer only pollen appear to offer higher quality pollen (e.g., a greater percentage of protein by mass) and more of it (Cruden 2000; Roulston et al. 2000). Like flowering plant species that offer nectar rewards, plant species that offer pollen rewards, including the 6-8% of angiosperm species that offer only pollen rewards, exhibit tremendous diversity in floral form, display features, and in reward quantity and quality (e.g., Vogel 1978; Faegri 1986; De Luca & Vallejo Marín 2013).

Plant species offering pollen rewards also show patterns of floral morphology that appear to reflect selection by pollen-foraging pollinators. For instance >6% of flowering plants species (>22,000 species spread across >80 families; Buchmann 1983; De Luca & Vallejo Marín 2013; S. Buchmann, D. Jolles & R. Kriebel unpub. data) conceal pollen within complex tube-like poricidal anthers or, less typically, corollas (Houston & Ladd 2002; De Luca & Vallejo-Marín 2013; Corbet et al. 2014). The recurrent evolution of such poricidal floral morphology is thought to reflect selection by generalist bees that extract the concealed pollen via a complex motor routine termed floral sonication (Buchmann 1983; De Luca & Vallejo Marín 2013).

Bumblebees are a model system for the study of generalist foraging ecology (e.g., Plowright & Laverty 1984). Most individuals within a bumblebee colony collect both pollen and nectar (O'Donnell et al. 2000; Hagbery & Nieh 2012), yet patterns of individual reward specialization and the mechanisms that account for such patterns have not been studied rigorously. In particular, it is an open question as to how patterns of pollen and nectar reward specialization might change over worker lifetime and whether differences in sensory morphology account for such patterns, as is thought to be the case for foraging honeybees (Riveros & Gronenberg 2010). In addition, whether and how generalist pollen-foraging bumblebees specialize on pollen-rewarding plant species is unknown. Much of the diversity of floral form amongst angiosperms is thought to be driven by pollinator preferences. Accordingly, the diversity of floral form amongst pollen-offering plant species suggests that pollen-foraging bees should exhibit floral preferences. Additionally, we might expect that effective pollen extraction from diverse

floral morphology (e.g., diverse anther forms) might require learning, especially when pollen must be extracted from complex floral morphology, comparable to learning of nectar extraction routines when floral nectar is concealed. Finally, by examining the mechanisms that account for floral specialization by pollen-foraging bumblebees, we may be better able to understand diverse patterns of floral trait evolution.

An explanation of the dissertation format

In this dissertation I investigated mechanisms of floral and reward specialization by generalist foraging bumblebees, *Bombus impatiens* Cresson, with a strong focus on the collection of pollen rewards. Generalist species of bumblebees forage for nectar on often dozens of diverse plant species that vary greatly in floral morphology (Plowright & Laverty 1984). The mechanisms by which these generalist pollinators effectively locate and use diverse floral resources have been much studied in the context of nectar collection. Yet scant work has examined the role of experience and floral features in shaping the effectiveness of pollen foraging behavior, and how individual bees manage collection of both pollen and nectar. My dissertation reveals that pollen foraging behavior has both similarities and fundamental differences to nectar foraging behavior. My work thus has fundamental implications for the evolution of floral traits and generalist bee foraging behavior.

The dissertation is presented in five appendices, each formatted as a manuscript. Appendix A examines patterns of daily and lifetime foraging effort and pollen and nectar reward specialization by all foragers within a colony and whether behavioral patterns

correspond to forager sensory morphology. Appendix B examines whether pollen-foraging bumblebees are able to learn preferences for plant species that offer only pollen as a floral reward, and the floral structures that mediate learned preferences. Appendix C examines the floral structures and sensory modalities that mediate the behavior of bumblebees foraging for concealed pollen, and how experience alters bee responses. Appendix D examines the role of experience in the expression of floral sonication by bumblebees foraging for concealed pollen rewards. Appendix E examines whether and how generalist bumblebees achieve efficient pollen collection on diverse floral resources.

PRESENT STUDY

The methods, results, and conclusions of this study are presented in the manuscripts appended to this dissertation. The following is a summary of the principal findings in this document.

Appendix A examines patterns of daily and lifetime foraging effort and pollen and nectar reward specialization by all foragers within a bumblebee colony and assesses how these patterns relate to forager sensory morphology. I obtained each forager's complete lifetime foraging history via radio frequency identification (RFID) technology. Each forager's body size and sensory morphology were obtained via digital microscopy. My results show that most bumblebees in a colony are lifetime generalists, but short-term specialists (>90% of daily bouts for one or the other reward). Specifically, most foragers specialize on either pollen or nectar over the course of a day, but switch from specializing on one reward to the other over longer periods of time. Furthermore, foraging effort (e.g., the number of bouts made per day) varies nearly 40-fold among foragers. Specialization is thought to increase task performance, yet short-term (over the course of a day) reward specialists did not make more foraging bouts. However, larger bees with more olfactory sensilla forage more and make more lifetime bouts for pollen.

Appendix B examines whether pollen-foraging bumblebees are able to learn preferences for plant species that offer only pollen as a floral reward, and the floral structures that mediate learned preferences. Using an absolute conditioning protocol, bees in this study were given experience collecting pollen from a single plant species. Bees' preferences for

flowers of that species relative to a second species were tested either one or 24 hours later. I find that the preference of experienced bees was shifted strongly towards the species experienced, relative to the preference of naïve bees. Results of the 24-hour test provide strong evidence of long-term memory. These changes in preference are best explained as associative learning. Additionally, while both corolla and anther responses are involved, anther responses are more strongly influenced by experience.

Appendix C examines the floral structures and sensory modalities that mediate the behavior of bumblebees foraging for concealed pollen, and how experience alters bee responses. Few studies have examined how pollen-offering flowers advertise their presence and, further, how they direct pollinators to pollen concealed within the anthers. This study examines the signaling function of two floral features in particular: the corolla and the anther. I find that visual features of the corolla advertise floral presence, while anther chemistry directs nearby bees to find and extract concealed pollen. Anther chemical cues serve as indirect cues of concealed pollen. Further, experience mainly modifies bees' responses to the anthers and anther chemistry. Both the anthers and corolla have important signaling functions for pollen-foraging bumblebees, as is the case for nectar-foraging bees. However, unlike for bees foraging for concealed nectar, bees foraging for concealed pollen rely mainly on anther cues as cues of concealed rewards.

Appendix D examines the role of experience in the expression of floral sonication by bumblebees foraging for concealed pollen rewards. Nectar foraging routines are typically learned, and cognitive constraints encourage pollinators to continue foraging on these

flowers (i.e., to exhibit floral fidelity). No studies have quantified the effect of experience on flower handling for bees extracting pollen from complex floral morphology. I therefore examine the degree to which floral sonication behaviour is modified by experience. The key elements of the sonication motor routine appear in full form in a flower-naïve bee's first visit to a flower. Additionally there are consistent, albeit modest, effects of experience on certain aspects of sonication behaviour. The latency to sonicate slightly decreases with experience. Bees also adjust the length and amplitude of their sonication buzzes in response to pollen receipt. We conclude that the complex sonication (pollen extraction) routine is expressed innately, unlike complex nectar extraction routines that are learned associatively (instrumental learning).

Appendix E examines whether and how generalist bumblebees achieve effective pollen collection on diverse floral resources. Bees scrabble for pollen when pollen is presented openly on the anthers; to collect pollen from flowers that conceal it within tubal floral morphology (poricidal floral morphology), bees sonicate. I characterize how generalist bumblebees (*Bombus impatiens*) forage effectively for pollen from diverse floral resources, by manipulating the presence of pollen and anther cues, in a series of experiments using pollen-bearing live flowers, flowers of a sterile pollenless horticultural hybrid, and artificial flowers. I demonstrate that bumblebees exhibit flexible and effective pollen collection by switching between their two pollen collection routines, floral sonication and scrabbling. Flexibility is regulated by the interplay between two ubiquitous floral cues: chemical anther cues stimulating sonication and mechanical pollen

cues suppressing it. This flexibility does not appear to involve instrumental learning, unlike flexibility exhibited during the collection of nectar from diverse floral resources.

REFERENCES

Anderson, B., Alexandersson, R., Johnson, S.D. 2009. Evolution and coexistence of pollination ecotypes in an African *Gladiolus* (*Iridaceae*). Evolution *6*, 960-972.

Buchmann, S.L. 1983. Buzz pollination in angiosperms. In Handbook of experimental pollination biology, Jones, C.E., Little, R.J., eds. (New York: Van Nostrand Reinhold). pp. 73–113.

Chittka, L. and Muller, H. 2009. Learning, specialization, efficiency and task allocation in social insects. Commun. Integr. Biol. *2*, 151-154.

Chittka, L., Thomson, J.D., Waser, N.M. 1999. Flower constancy, insect psychology, and plant evolution. Naturwissenschaften *86*, 361–377.

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

Corbet, S.A. and Huang, S.Q. 2014. Buzz pollination in eight bumblebee-pollinated *Pedicularis* species: does it involve vibration-induced triboelectric charging of pollen grains? Ann. Bot. *114*, 1665-1674.

Cruden, R.W. 2000. Pollen grains: why so many? Plant Syst. Evol. *222*, 143-165.

Dornhaus, A. 2008. Specialization does not predict individual efficiency in an ant. *PLoS Biol*. http://dx.doi.org/10.1371/journal.pbio.0060285

Faegri, K. 1986. The solanoid flower. Trans. Bot. Soc. Edinburgh *45*, 51-59.

Foster, J.J., Sharkey, C.R., Gaworska, V.A., Roberts, N.W., Whitney, H.M., Partridge, J.C. 2014. Bumblebees learn polarization patterns. Curr. Biol. *24*, 1415-1420.

Gegear, R.J., Burns, J.G. 2007. The birds, the bees, and the virtual flowers: can pollinator behavior drive ecological speciation in flowering plants. Am. Nat. *170*, 551-556.

Gegear, R.J., Laverty, T.M. 1995. Effect of flower complexity on relearning flower-handling skills in bumble bees. Can. J. Zool. *73*, 2052-2058.

Giger, A.D., Srinivasan, M.V. 1995. Pattern recognition in honeybees: eidetic imagery and orientation discrimination. J. Comp. Physiol. A *176*, 791-795

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.

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

Hagbery, J. and Nieh, J.C. 2012. Individual lifetime pollen and nectar foraging preferences in bumble bees. *Naturwissenschaften 99*, 821-32.

Hopkins, R. and Rausher, M.D. 2012. Pollinator-mediated selection on flower color allele drives reinforcement. Science *335*, 1090-1092.

Hopkins, R. and Rausher, M.D. 2014. The cost of reinforcement: selection on flower color in allopatric populations of *Phlox drummondii*. Am. Nat. *183*, 693-710.

Houston, T.F. and Ladd, P.G. 2002. Buzz pollination in the Epacridaceae. Aust. J. Bot. *50*, 83-91.

Kevan, P.G., Baker, H.G. 1983. Insects as flower visitors and pollinators. Annu. Rev. Entomol. *28*, 407-453.

Laverty, T.M. and Plowright, R.C. 1988. Flower handling by bumblebees: a comparison of specialists and generalists. Anim. Behav. *36*, 733-740.

Laverty, T.M. 1994. Bumble bee learning and flower morphology. Anim. Behav. *47*, 531-545.

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

Lewis, A.C. 1993. Learning and the evolution of resources: pollinators and flower morphology. In: Papaj, D.R., Lewis, A.C., editors. Insect learning: ecological and evolutionary perspectives. New York: Chapman & Hall. pp. 219-242.

Menzel, R. 2012. The honeybee as a model for understanding the basis of cognition. Nature Rev. *13*, 758-768.

Nicolson, S.W. 2011. Bee food: the chemistry and nutritional value of nectar, pollen and mixtures of the two. Afr. Zool. *46*, 197-204.

O'Donnell, S., Reichardt, M. and Foster, R. 2000. Individual and colony factors in bumble bee division of labor (*Bombus bifarius nearcticus* Handl; Hymenoptera, Apidae). Insectes Soc. *47*, 164-170.

Page, R.E., Scheiner, R., Erber, J. and Amdam, G.V. 2006. The development and evolution of division of labor and foraging specialization in a social insect (*Apis mellifera* L.). Curr. Top. Dev. Biol. *74*, 253-286.

Page, Jr., R.E., Linksvayer, T.A. and Amdam, G.V. 2009. Social life from solitary regulatory networks: a new paradigm for insect sociality. In: Gadau, J., Fewell &

J.H. editors. Organization of Insect Societies: From Genomes to Socio-complexity. Massachusetts: Harvard University Press. pp. 355-374.

Papaj, D.R. and Lewis, A.C. 1993. Insect learning: ecological and evolutionary perspectives. New York: Chapman & Hall.

Plowright, R.C. and Laverty, T.M. 1984. The ecology and sociobiology of bumble bees. Annu. Rev. Entomol. *29*, 175-199.

Raine, N.E. and Chittka, L. 2007. Pollen foraging: learning a complex motor skill by bumblebees (*Bombus terrestris*). Naturwissenschaften *94*, 459-464.

Riveros, A.J. and Gronenberg, W. 2010. Sensory allometry, foraging task specialization and resource exploitation in honeybees. Behav. Ecol. Sociobiol. *64*, 955-966.

Roulston, T.H., Cane, J.H., Buchmann, S.L. 2000. What governs the protein content of pollen grains: pollinator preferences, pollen-pistil interactions, or phylogeny. Ecol. Monog. *70*, 617-643.

Schiestl, F.P. and Johnson, S.D. 2013. Pollinator-mediated evolution of floral signals. Trends. Ecol. Evol. *28*, 307-315.

Simpson, B.B. and Neff, J.L. 1981. Floral rewards: alternatives to pollen and nectar. Ann Mo Bot Gard. *68*, 301-322.

Vogel S. 1978. Evolutionary shifts from reward to deception in pollen flowers. In: Richards, A.H., editor. The pollination of flowers by insects, London: Academic Press. pp. 89-96.

Waser, N.M. 1986. Flower constancy: definition, cause and measurement. Am. Nat. *127*, 593-603.

Westerkamp, C. 1999. Keel flowers of the Polygalaceae and Fabaceae: A functional comparison. Bot. J. Linn. Soc. *129*, 207-221.

Whitney, H.M., Kolle, M., Andrew, P., Chittka, L., Steiner, U., Glover, B.J. 2009. Floral iridescence, produced by diffractive optics, acts as a cue for animal pollinators. Science *323*, 130-133.

APPENDIX A

PATTERNS OF POLLEN AND NECTAR FORAGING SPECIALIZATION BY BUMBLEBEES OVER MULTIPLE TIMESCALES USING RFID

Full Title: Patterns of pollen and nectar foraging specialization by bumblebees over multiple timescales using RFID

Avery L. Russell[a*], Sarah J. Morrison[b], Eleni H. Moschonas[c], and Daniel R. Papaj[c]

[a] Graduate Interdisciplinary Program in Entomology and Insect Science, University of Arizona, Tucson, AZ, 85721. USA

[b] Lunar and Planetary Laboratory, University of Arizona, Tucson, AZ, 85721. USA

[c] Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, 85721. USA

*Corresponding author

This manuscript has been submitted to *Scientific Reports*.

ABSTRACT

The ecological success of social insects is frequently ascribed to improvements in task performance due to division of labour amongst workers. While much research has focused on improvements associated with lifetime task specialization, members of colonies can specialize on a given task over shorter time periods. Eusocial bees in particular must collect pollen and nectar rewards to survive, but most workers appear to mix collection of both rewards over their lifetimes. We asked whether bumblebees specialize over timescales shorter than their lifetime. We also explored factors that govern such patterns, and asked whether reward specialists made more foraging bouts than generalists. To address all of these issues, we described antennal morphology and size of all foragers in a colony and related these factors to each forager's complete foraging history, obtained using radio frequency identification (RFID). Only a small proportion of foragers were specialists over their lifetime; nevertheless, >50% of foragers specialized daily on a given reward. Contrary to expectations, short and long-term reward specialists were not the best foragers (being neither larger nor making more bouts); larger bees with more antennal olfactory sensilla were better foragers, but were not more specialized. We discuss causes and functions of short and long-term patterns of specialization for bumblebee colonies.

INTRODUCTION

Task specialization is a hallmark of insect societies[1,2]. Eusocial bees must engage in a variety of tasks over their lifetime, including nest construction, brood care, and foraging from flowers to feed themselves and their nest mates. Specialization on different foraging tasks in particular has been well studied (e.g.,[3,4]). Because switching between tasks can incur temporal, cognitive, and/or energetic costs, specialization is thought to maximize task efficiency[3,5,6]. For instance, bees often have to learn new nectar collection routines each time they shift to a new plant species[3,7]. Cognitive costs associated with learning and recalling these nectar collection routines are thought to make it advantageous for nectar-foraging bees to specialize in the short term on a given plant species[3,7,8]. In addition, individuals can vary in their task performance as a result of physiological or morphological differences; for example, honeybees vary in their sucrose sensitivity[4,9]. In fact, intrinsic differences among foragers are thought to explain lifetime patterns of specialization on the collection of nectar, water, and pollen in foraging honeybees[9,10]. These and other studies suggest that patterns of foraging task specialization by individual worker bees might vary when we examine the short term (e.g., hours or days) versus the long term (e.g., lifetime). Yet, almost no research has examined specialization by the same foragers over different timescales.

Patterns of foraging specialization over different timescales have important implications for how individuals and, for social bee species, colonies manage the collection of multiple floral rewards. The two most common floral rewards collected by bees are

pollen and nectar[11,12]. Flowering plants offer pollen and nectar in all combinations and qualities. For instance, the flowers of some plant species offer pollen and nectar, only pollen, only nectar, or even vary the availability of one or the other reward over the floral life cycle or as a result of prior collection (Fig. 1a-d). Thus, a forager might not always be able to collect pollen and nectar on a single floral visit (e.g.,[13,14]). Further, collecting both rewards during a single foraging bout may not be efficient if, for instance, the bee has to travel far to collect both rewards. In addition, colony needs vary over lifetime. Bees rely on nectar as their primary source of carbohydrates and pollen as their primary source of protein and lipids[15,16]. While worker bees primarily consume nectar, larvae consume prodigious amounts of pollen[16,17]. Changes in the amount of brood (e.g.,[18]) and in storage of one or the other nutrient (e.g.,[12,17,19]) can therefore change the relative need for each resource. Given the dynamic nature of the resource environment and nutrient demands of individuals and the colony, patterns of individual reward specialization (i.e., the ratio of pollen to nectar collection bouts a given forager makes) might be expected to change over time.

Apart from a relative dearth of information about patterns of reward specialization, we know even less about the mechanistic factors that govern reward specialization. However, one body of literature ascribes variation in reward specialization to intrinsic individual differences in behaviour and sensory properties. According to the well-supported response threshold model (RTM), reward specialization arises as a result of interindividual variation in sensitivity to cues that stimulate nectar or pollen foraging (so called ‘response thresholds’[9,10]). In honeybees, reward specialization and corresponding

variation in response thresholds are associated, at least in part, with variation in sensory structures[10]. Specifically, differences in the number of antennal olfactory sensilla (i.e., sensilla placodea) correspond to differences in reward specialization, independent of variation in body size[10]. In particular, honeybee workers with low response thresholds (high sensitivity) typically forage for pollen, whereas workers with high response thresholds (low sensitivity) typically forage for nectar[9,10].

In this study, we assessed patterns of individual reward specialization in bumblebees, and the possible role of sensory morphology in driving these patterns. Relative to other eusocial bee species, bumblebees (*Bombus*) exhibit considerably within-colony variation in body size (i.e., alloethism). Large body size is thought to be associated with greater foraging efficiency; larger bees of a given species have better sensory systems for detecting and assessing flowers (e.g., allometric scaling results in bigger eyes and more antennal olfactory sensilla), can carry more nutrients before returning to the nest (e.g., bigger storage organs), and can forage further afield[20,21,22,23]. Yet, any association of body size and reward specialization remains ambiguous. We predict that larger bumblebee workers, which should have low response thresholds due to greater numbers of sensilla placodea, will be more specialized on pollen. Alternatively, we might expect that specialists on either reward would be larger and thus more effective foragers; sensilla placodea number might instead relate more strongly to the degree to which a forager is specialized on a given reward.

To test these hypotheses, we compiled a complete lifetime record of pollen and nectar foraging activity for every bumblebee forager (*Bombus impatiens*) in a colony and related these patterns to the sensory morphology and size of each forager. We used radio frequency identification (RFID) technology to track bees as they moved between foraging chambers that presented either only pollen or only nectar from artificial flowers. We measured antennal sensory morphology (sensilla placodea number or 'pore plates') and other morphological attributes (forewing length, head width, antennal length, and proboscis length) which might be associated with different patterns of reward specialization and foraging effort. This is the first study to our knowledge that examines patterns of reward specialization at multiple timescales, and that connects them to forager size and sensory morphology.

METHODS

Bees

We used a colony of *Bombus impatiens* in a study conducted between September 2013 and January 2014. The 4-week old colony was purchased from Koppert Biological Systems (Howell, MI, USA) and had 12 workers when it arrived. The colony was allowed to acclimate until new foragers began to eclose 23 days later, whereupon the colony was attached to the experimental foraging setup described below. During acclimation, bees were trained to forage for pollen and nectar in a foraging arena (LxWxH 58 x 73 x 40 cm) that provided *ad libitum* purchased honeybee-collected pollen

(Koppert Biological Systems, MI, USA) and 1M sucrose solution. A single feeder dispensed sucrose solution via braided cotton wicks (6 inch Braided Cotton Rolls, Richmond Dental) that extended into 40 dram vials through perforations made in the white plastic lids (BioQuip Products, Inc.). Pollen was presented from a single custom-made feeder[24] consisting of chenille fibres (white to the human eye), glued to the inside walls of 40 dram vials (BioQuip Products, Inc., USA). Beside the natural scent or colour of the sucrose solution or pollen, neither feeder was scented or coloured.

After the colony was attached to the experimental foraging setup, bees that died were frozen at -18C and stored in individually labelled BEEM capsules (Ted Pella, Inc.). Two dead bees were too damaged for use in subsequent morphological analyses.

Experimental foraging setup

An arena constructed of plywood (LxWxH 58 x 73 x 40 cm) was divided into two equal-sized foraging chambers (each LxWxH 58 x 36 x 40 cm) via a removable plywood wall (Fig. 2a). The floor and sides of the arena were painted grey. The arena had a clear acrylic ceiling and was lit from above by 40W 60Hz fluorescent lights (Lithonia Lighting). The colony was kept dark while lights in the arenas were set to a 12:12 light:dark cycle.

One entrance hole was drilled through the outer wall of each foraging chamber (Fig. 2b). The colony box (accessible via a single hole) was joined to the foraging chambers by a

branching tunnel with ends attached to each chamber (Fig. 2d). Two RFID readers (MAJA reader module 4.2, Microsensys GmbH, GE) were mounted in sequence on custom holders at the entrance of each foraging chamber (Fig. 2c). As bees moved through the paired readers, the identity of the chamber and direction of movement (into versus out of the chamber) was automatically recorded. RFID readers in a pair were held 3cm apart using foam: readers with less separation fail to read transponders. To reduce the chance of readers failing to detect tagged bees, the floor of each reader's tunnel (through which the bee traversed and was scanned) was slightly elevated to bring bees closer to the readers, using custom built plastic pieces (LxWxH 8 x 2 x 1.1 mm) taped in place.

One chamber provided *ad libitum* honeybee-collected pollen and the other provided 1M sucrose solution. Three feeders dispensed sucrose solution and three feeders dispensed pollen. Feeders were of the design previously described. Feeders were suspended 17cm from the floor of the chamber (measured from the top of the feeder) by way of custom-built holders (Fig. 2a). This design presented a more realistic foraging situation by preventing those bees that could not (or would not) fly from foraging.

Tagging procedure

Unlabelled bees that left the colony box were tagged with 1.5 x 1.0 x 0.5 mm RFID transponders (mic3-tag 64 RO, Microsensys GmbH, GE). Each transponder ('tag') weighed 2.5 mg, approximately 2.6% of the weight of a bee (mean bee weight in mg for

this colony ± SE: 95.5 ± 4.7), 4.9% of a nectar load[25], and 15% of a pollen load (A. Russell unpub. data). To find foragers that had not yet been tagged, we checked the foraging arena and tunnels every 15-60 minutes, 8 hours each day, 6 days a week (Monday-Saturday). Unlabelled foragers were briefly trapped in a part of the tunnel that had a removable bottom and thereby captured in 40 dram vials (BioQuip Products, Inc.). Foragers were immobilized in a Queen Marking Tool (The London Bee Company Ltd, Middlesex, UK) and a single tag glued to the dorsum of their thoraxes with a small amount of superglue. Shortly (<1 minute) thereafter, bees were individually placed into vials with pollen scent to calm them (this eliminated aggression by other bees to the newly-labelled bee; A. Russell pers. obs.), then transferred back into the colony after approximately 2 minutes. As bees aged, wax built up on their tags. As this extra mass could potentially impede their foraging efficiency, we gently (without disturbing the other bees) extracted bees from the colony once a week during night-time hours and scraped the wax off with forceps.

Morphological measures

On each bee, we measured head width, forewing length, proboscis length, antennal flagellum length, the 7th antennal flagellomere's length, and the width and number of pore plates. It took 15 months to measure all bees. Measurements were done as follows. The head of each bee was first removed and photographed in frontal view at 1.2X using a digital camera with a 5.2 mega pixel resolution (DCM500 Microscope CMOS Camera, Microscope Cameras), affixed within the ocular of a stereoscope. Next, the proboscis

(proximal base of postmentum to distal tip of labellum), antennae (at the scape), and forewings (without damaging the basal articular sclerites) were removed. The antennae were flat-mounted and photographed at 2X via the digital camera setup. The proboscis and forewings were sealed between two clear transparencies (Staples Transparency Copy Film, Staples, Inc.) using mounting medium and then scanned at 2400dpi (Epson Perfection 3490 Photo, Epson America, Inc.). ImageJ (National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/) and a micrometre were used to make and calibrate all measures, respectively. Head widths were measured at their widest point. Forewing length was measured from the distal edge of the marginal cell (the distal-most leading vein at the point where R1 and R meet; Fig. 3a), to the base of the radius, at which point it meets the sclerite. Proboscis length was measured from proximal base of prementum to distal tip of labellum (Fig. 3b). We measured the centre-line length of the antennal flagellum. In addition, the width and length of the 7th antennal segment were measured and the surface area of the structure calculated, assuming a cylinder.

To count sensillae, antennae were coated in a thin layer of clear nail polish (Hard as Nails, Sally Hansen) and suspended in air for 3 minutes to dry. Antennal casts were cut open lengthwise with a scalpel, making sure to cut only through the area of the antennae that exhibited hair plates (sensilla trichodea) and not pore plates (sensilla placodea) (Fig. 3c). Casts were peeled off and mounted flat between a slide and coverslip so that we could examine the complete surface of the antenna in one focal plane. We focused on the pore plates (sensilla placodea) because of their established function in olfaction[26] and because they differ between honeybee pollen and nectar foraging specialists[10]. Because

our methodology did not produce intact casts for all antennal segments, we made drawings of the casts of antennal segment 7 (segments 8-10 were sometimes damaged) and all its pore plates at 10X magnification, via a camera lucida mounted to a compound microscope (Leitz Laborlux). We focused on this distal segment because pore plates are more numerous on more distal antennal segments[28] and differences amongst bees might therefore be more readily quantified. The pore plate density on the 7th segment was calculated using our ImageJ measurements.

There were small differences between forewings in a pair (<3%) and between antennae in a pair (<7%), but there was no overall bias across bees for one particular side for either forewings or antennae (Supplementary Materials); thus, when left and right measures were available, we always used the larger of the two.

Calculating the amount of brood

The colony was photographed through the Plexiglas cover from above each day. We used a custom-built stand to hold the camera at constant height and location. Using ImageJ we traced the perimeter of the colony (the wax comb) and the perimeter of the brood cells (completely closed cells). ImageJ's 'area' function was used to determine the percent of the colony's surface composed of brood cells. Because young colonies tend to grow outward, on the surface of the nest box, rather than upward, as older colonies tend to do (A. Russell pers. obs.), this measurement of brood is likely relatively accurate.

RFID data processing software

Custom written MATLAB R2013b software was used to process RFID data and extract foraging related parameters. Throughout this manuscript we define a 'foraging bout' (or 'bout') as a bee entering a foraging chamber and staying within it for more than 60 seconds, but less than 30 minutes. For a subset of the data (days 1-5), 98.6% of visits within the foraging chamber lasted less than 30 minutes (mean bout duration in minutes ± SE: 6.98 ± 0.15). Additionally, we visually confirmed on days 1-5 that 100% of bees that spent less than 60 seconds in the flight arena did not forage (they entered and then left the arena rapidly; mean length of all visits in seconds for days 1-5 = 22.4 seconds) and that approximately 80% of bees in the foraging chamber were actually collecting sucrose (proboscis extended and abdomen pumping on the sucrose feeder) or pollen (bee packing pollen into its pollen baskets) at any given time.

A foraging bout (or 'bout') was assigned using the following method. An 'in' (IN) event was scored as the detection of an RFID tag by the outermost reader (distal to the colony box), of the pair attached to each foraging chamber, followed by a detection of that same tag by the innermost reader (proximal to the colony box). Conversely, an 'out' (OUT) event was scored as a detection from the innermost reader followed by that from the outermost reader. We set a threshold time difference value between inner and outer reader detections ('pairing threshold') to score whether detections were paired (and therefore constituted a bee leaving or entering the nest as part of a foraging bout). If a bee took longer than 60 seconds to pass through the two readers, the two different reader

detections were scored as unpaired (occasionally bees will stop between readers, leading to this problem).

A complete foraging bout consists of a bee generating an OUT event followed by an IN event, thus necessitating 4 detections in total (2 detections for each reader pair). The failure to record any of these 4 required detections disqualified the putative bout from further analysis. We scored foraging bouts over the lifetime of each bee. The first day of foraging for each bee were excluded when examining patterns of individual foraging behaviour to ensure our dataset only included days during which bees could forage throughout the day. The duration of a given foraging bout was calculated by determining the time elapsed between paired OUT and IN events (with the average time point of paired reader detections serving as start and end points for a foraging bout).

Each RFID tag can be read multiple times as a bee passes through a reader. We set a threshold time difference value for combining sequential same-tag detections ('same bout threshold'). This threshold also affects the number of unpaired detections. For instance, if a bee switches from travelling into the colony to going out of the colony 15 seconds after the last detection from the same reader, a second bout was counted. If a bee took less than 15 seconds to make this manoeuvre, no second bout was counted. For a subset of the data (days 1-5) 96.5% of the 82609 sequential same-tag detections that occurred fell below this 15-second threshold. The mean same-tag detection interval was 4.16 ± 0.26 seconds; the median was 0.184 seconds.

Reader failure rate was estimated for 1 hour of observations for each of 5 consecutive days, involving 79 bouts. Zero of these events failed to be recorded by either reader, while 77 involved only one reader reporting a tag. Thus 3% of all interactions failed to report a foraging bout, resulting in a reader failure rate similar to that reported in other studies[28,29]. We assumed reader failures occurred with the same frequency for each bee.

Data analyses

All data were analysed using R v.3.2.0[30].

We tested for a significant association between degree of reward specialization and lifetime foraging period for foragers using a linear model (LM). Possible values range from 0.5 to 1: values closer to 0.5 belong to bees that collected both rewards equally frequently; values closer to 1 belong to bees that were specialized on a given reward (either pollen or nectar). The LM was specified using the lm() function in R. 'Degree of reward specialization' was specified as the independent variable and 'lifetime foraging period' was specified as the response variable. We log transformed the independent variable and thereby normalized the residuals.

To describe variation in daily degree of reward specialization over forager lifetime, we applied a Shannon Diversity Index, which accounts for both abundance and evenness of the variation[31]. The Shannon Diversity Index (H) as applied to our data was calculated as $H = -\sum pi \ln(pi)$, where 'p_i' is the proportion of nectar bouts made for a given day,

multiplied by the natural log of this proportion ($\ln p_i$). For each forager, this value is summed across all foraging days and multiplied by -1. Bees with less flexibility in their reward specialization across days over lifetime have values closer to 0. We used a one-way Wilcoxon signed rank test to determine whether bees on average showed flexibility in daily degree of reward specialization.

To determine how foragers switched between foraging for pollen versus nectar across bouts while controlling for any bias in their reward preference, we applied a modified Jacob's Constancy Index[32], calculated as $CI = \frac{c - e}{c + e - 2ce}$, where 'c' was the proportion of transitions between the same reward (i.e., the proportion of sequences made to one reward following an immediately prior bout made to that same reward) and where 'e' was the proportion of transitions between the same reward given the expected proportion (the overall frequency of nectar bouts over the lifetime of the forager). Possible values range from -1 to 1: bees that were more inconstant (systematically alternated between rewards) have values closer to -1; bees that made random transitions between rewards have values closer to 0; bees that were more constant (i.e., foraged in runs for one or the other reward) have values closer to 1. We used a one-way Wilcoxon signed rank test to determine whether bees on average foraged in runs.

The 14 bees that showed complete constancy (CI = 1) or complete inconstancy (CI = -1) made very few bouts over their lifetime (18 or fewer bouts, as compared to a colony-wide mean ± SE of 115.6 ± 15.3 bouts over forager lifetime; N = 98 foragers), raising the possibility that these CI values were a result of small sample size. We therefore discarded

all bees that had made 18 or fewer bouts from this analysis (22 total bees). Mean Jacob's CI dropped as a result (mean ± SE: before, 0.18 ± 0.04; after, 0.11 ± 0.03).

We ran a Chi-square (χ^2) test via the chisq.test() function in R to analyse whether lifetime reward specialists made proportionally fewer bouts. Likewise, we ran a paired *t*-test via the t.test() function in R to analyse whether daily reward specialists made proportionally fewer daily bouts.

We initially used multivariate multiple regression models (MMRs) to determine whether morphological characteristics were associated with behavioural patterns. MMRs were specified via the lm() function in R. We used multiple MMRs to retain power and eliminate errors; we therefore grouped variables in MMRs by similarity (lifetime variables grouped in one MMR, daily mean variables grouped in a separate MMR). With one MMR we examined effects on lifetime behaviour ('total days foraged', 'lifetime bouts', 'lifetime nectar bouts', 'lifetime pollen bouts', 'lifetime nectar preference'). For this MMR we log transformed 'lifetime bouts', 'lifetime nectar bouts', 'lifetime pollen bouts' and thereby normalized the residuals. A second MMR was used to determine effects on mean daily behaviour ('mean daily bouts', 'mean daily nectar bouts', 'mean daily pollen bouts'). For this second MMR we log transformed the response variables and thereby normalized the residuals. Morphological characteristics ('forewing length', 'head width', 'proboscis length', 'pore plate number', 'pore plate density') were specified as independent variables, while behavioural characteristics were specified as response variables via the cbind() function in R. To simplify these MMRs we applied backwards

elimination via the mStep() function in the qtlmt package[33]. For the first MMR all independent variables aside from 'pore plate number' were eliminated; for the second MMR all independent variables aside from 'forewing length' were eliminated. We subsequently report results of linear models (LMs) for each separate dependent variable, applying a conservative Bonferroni correction (α-value = 0.006). We discarded 22 bees that had zero values in at least one category (otherwise we encountered errors).

In addition, we used separate LMs to determine effects of colony age on brood and foraging characteristics, applying a Bonferroni correction (α-value = 0.017). Colony age was specified as the independent variable for each LM. Daily 'percent brood', 'colony reward preference', 'foraging force size', 'mean bouts per forager', percentage of daily 'reward specialists' (>90% of bouts per day for either nutrient), percentage of daily 'nectar specialists', or percentage of daily 'pollen specialists' were specified as the response variable. For the LM examining the effect of colony age on the size of the active foraging workforce (foraging force size) we used an *F*-test via the anova() function in R to test whether a quadratic model was better than a linear model. The quadratic model was a much better fit ($F_{1,40} = 74.272$, $P < 0.0001$, $N = 43$ days) and we therefore only report the results of this quadratic model. For the LM examining the effect of colony age on daily percent brood, we discarded 10 days for which photos of brood analysis were not available (due to file corruption); missing days were distributed approximately evenly.

We also used an LM to determine whether forager lifetime nectar preference was associated with mean daily measures of foraging performance. Log-transformed

measures of foraging performance ('daily nectar bouts' and 'daily pollen bouts') were specified as fixed factors and forager lifetime nectar preference was specified as the dependent variable. To examine the possible significance of an interaction between daily nectar bouts and daily pollen bouts, results were first examined using type III sums of squares via the 'Anova()' function in the car package[34]. Because the interaction was not statistically significant, we report results with a Type II ANOVA via the 'Anova()'. In a separate LM, we used the offset() function in R to change the null hypothesis of the linear regression to a slope of 1.We discarded 14 bees that had zero values in at least one category (otherwise we encountered errors).

We used a linear mixed effects model (LMM) to determine the effect of forager age on the number of bouts made for each forager per day. 'Forager age' was specified as the independent variable and also included as a repeated measures factor within the random factor 'BeeID'. The model was specified via the lmer() function in the lmerTest package[35]. We report the overall result via type II Wald chi-square ($\chi 2$) tests, using the Anova() function.

To determine whether morphological characteristics ('forewing length', 'head width', 'proboscis length', 'pore plate number', 'antennal length', '7^{th} antennal segment width', '7^{th} antennal segment length', and 'pore plate density') were correlated with one another via Pearson's *r* we used the cor() function in R. Correlations between lifetime behavioural measures ('total days foraged', 'lifetime bouts', 'lifetime nectar bouts',

'lifetime pollen bouts', 'lifetime nectar preference') were assessed in the same way. Correlations are reported in the Supplementary Materials.

RESULTS

We obtained a complete foraging record for the growth phase of the colony (43 days; from eclosion of the first new workers to eclosion of the first reproductives, marking the end of worker replacement and colony growth). Of 111 RFID-tagged bees, 103 made at least one foraging bout after being tagged. The colony made 11507 foraging bouts (mean per day: 620.3 bouts); 7801 for nectar (mean bout length in minutes ± SE: 7.62 ± 0.06) and 3706 for pollen (mean bout length in minutes ± SE: 5.64 ± 0.09).

Most foragers were reward generalists over lifetime, but specialists in the short term

There was a continuous distribution of lifetime reward preference among foragers (Fig. 4a). Only a minority (12.4%) of foragers could be classified as long-term reward specialists (making >90% of their foraging bouts for a single type of reward over their lifetime[1,36]) on either nutrient. Degree of lifetime reward specialization, ranging from 1 (completely specialized on a given reward) to 0.5 (collecting both rewards equally frequently) was not significantly associated with lifetime foraging period (LM: $F_{1,96}$ = 2.92, P = 0.091, R^2 = 0.019).

Conversely, short-term reward specialization was common: on average, 51.1% of a day's foragers made >90% of their bouts for a single type of reward (Fig. 4b; see Fig. 4e top and bottom traces for examples). Many of these foragers switched between specializing on either pollen or nectar over a day or more, thereby mixing rewards over their lifetimes (see Fig. 4e middle trace for an example). Additionally, most bees exhibited substantial flexibility in their daily degree of reward specialization: the mean Shannon Diversity Index (*H*) value for all foragers over lifetime was significantly greater than zero (no flexibility) (Fig. 4g; one-way Wilcoxon signed rank test: $V = 3321$, $P < 0.0001$, $N = 103$ bees; mean $H \pm$ SE: 1.97 ± 0.21). Despite mixing rewards, most bees foraged in runs for one or the other reward: the mean Jacob's Constancy Index (CI) value for all foragers over lifetime was significantly greater than zero (Fig. 4f; one-way Wilcoxon signed rank test: $V = 1858$, $P < 0.0001$, $N = 68$ bees; mean Jacob's CI $\pm$ SE: 0.11 ± 0.03). Likewise, most foragers (73.5% of 68 foragers) had a CI > 0.

High activity foragers contributed disproportionately to foraging effort

Mean daily foraging effort, estimated as mean number of daily foraging bouts, varied nearly 40-fold among foragers (Fig. 5a). In fact, half of the colony's mean number of daily foraging bouts were performed by a minority (17.3%) of foragers (high activity foragers, mean daily bouts $\pm$ SE: 20.7 ± 2.2, $N = 17$ bees). Conversely, when foragers were ranked by activity level, the bottom 50% made just 16.7% of the colony's daily foraging bouts (low activity foragers, mean daily bouts $\pm$ SE: 2.4 ± 0.1, $N = 49$ bees).

Variation in mean daily nectar foraging effort was more substantial than variation in mean daily pollen foraging effort (Fig. 5c).

Importantly, short (daily) and long-term (lifetime) reward specialists (foragers that made >90% of their bouts for a single type of reward for the given duration) contributed little to foraging effort: long-term reward specialists (12.4% of 97 total foragers) made just 6.3% of all foraging bouts made by the colony (Fig. 4a,c); short-term specialists (51.1% of 33.1 ± 1.4 foragers on average) likewise made only 33.9% of mean daily foraging bouts (Fig. 4b,d). Proportionally, daily reward specialists made significantly fewer foraging bouts each day given the expectation these proportions would be the same (paired *t*-test: % of bouts made by reward specialists each day versus % of foragers each day that were reward specialists, $t_{42} = 10.991$, $P < 0.0001$, $N = 43$ days). Although not significant, lifetime reward specialists also tended to make fewer foraging bouts (χ^2-test: % bouts made by lifetime reward specialists versus % of foragers that were lifetime reward specialists, $\chi^2 = 1.99$, $P = 0.158$).

High activity foragers preferred to collect nectar over lifetime

Forager preference to collect nectar was strongly associated with mean daily number of bouts made for nectar and pollen (Fig. 6a; LM overall effect: $F_{3,85} = 1429$, $P < 0.0001$, $R^2 = 0.98$, $N = 89$ bees). Specifically, increasing preference to collect nectar was strongly positively associated with more daily nectar bouts and, to a lesser degree, negatively associated with more daily pollen bouts (Fig. 6a; Type II ANOVA: log(daily nectar

bouts) effect: $F_{1,85}$ = 4062.1, P < 0.0001; log(daily pollen bouts) effect: $F_{1,85}$ = 1847.8, P < 0.0001). There was no significant interaction between mean daily nectar bouts and mean daily pollen bouts (Type II ANOVA: daily bouts:daily nectar bouts effect: $F_{1,85}$ = 0.2817, P = 0.597). Further, as foragers made more mean daily bouts, they tended to become less specialized on nectar collection (Fig. 6b; Type II ANOVA: slope <1 for LM of log(daily nectar bout) on log(daily pollen bout): $F_{1,87}$ = 76.722, P < 0.0001).

Effects of colony age on reward preference, specialization, and amount of brood

Amount of brood, colony reward preference, and the size of the active foraging workforce changed significantly over the lifetime of the colony (N = 43 days; Fig. 7a-d). Specifically, as the colony aged, the amount of brood (percent colony surface composed of brood cells) declined significantly and the colony shifted to foraging significantly more for nectar relative to pollen (Fig. 7a-b; LM: age effect on: percent brood, $F_{1,31}$ = 42.82, P < 0.0001, R^2 = 0.580; colony reward preference, $F_{1,41}$ = 5.838, P < 0.014, R^2 = 0.139; Bonferroni correction α-value = 0.017). Furthermore, as the colony aged, the active foraging workforce increased significantly and the mean daily foraging bouts per forager decreased significantly (Fig. 7c-d; LM: age effect on: foraging force size, $F_{1,41}$ = 59.85, P < 0.0001, R^2 = 0.737; mean bouts per forager, $F_{1,41}$ = 25.89, P < 0.0001, R^2 = 0.387; Bonferroni correction α-value = 0.017), even while a forager's mean daily foraging rate did not change significantly with forager age (LMM: forager age effect, χ^2 = 0.708, P < 0.401, N = 103 bees).

Patterns of reward specialization also changed significantly over the lifetime of the colony (N = 43 days; Fig. 7e-g). Specifically, as the colony aged the percentage of bees that specialized daily on pollen significantly decreased, while there was a trend for the percentage of bees that specialized daily on nectar to increase (Fig. 7e-g; LM: age effect on: % pollen specialists, $F_{1,41}$ = 8.124, P < 0.007, R^2 = 0.165; % nectar specialists, $F_{1,41}$ = 3.555, P = 0.066, R^2 = 0.080; Bonferroni correction α-value = 0.017). There was no significant correlation with percentage of daily reward specialists (>90% of bouts per day for either nutrient) (Fig. 7e-g; LM: age effect on: % reward specialists, $F_{1,41}$ = 0.207, P = 0.651, R^2 = 0.005; Bonferroni correction α-value = 0.017)

Bees with more pore plates foraged more for pollen

Bees with more antennal pore plates foraged for more days and made more pollen bouts over their lifetime (Fig. 8a-c; LM: pore plate number effect on: total days foraged, $F_{1,79}$ = 17.52, P < 0.0001, R^2 = 0.182; log(lifetime bouts), $F_{1,79}$ = 7.18, P < 0.009, R^2 = 0.083; log(lifetime pollen bouts), $F_{1,79}$ = 12.4, P < 0.0008, R^2 = 0.136; Bonferroni correction α-value = 0.006; pore plate number varied by a factor of 2.09 across all foragers). There was no significant association between pore plate number and a forager's lifetime number of nectar bouts or the degree to which a forager preferred to collect nectar over its lifetime (Fig. 8d-e; LM: pore plate number effect on: log(lifetime nectar bouts), $F_{1,79}$ = 3.97, P = 0.05, R^2 = 0.048; lifetime nectar preference, $F_{1,79}$ = 0.518, P = 0.474, R^2 = 0.007; Bonferroni correction α-value = 0.006).

Forewing length (a proxy for body size) was not associated with mean daily number of bouts (for pollen, nectar, or both) (LM: forewing length effect on: log(daily pollen bouts), $F_{1,79} = 6.54$, $P < 0.013$, $R^2 = 0.076$; log(daily nectar bouts), $F_{1,79} = 0.807$, $P = 0.372$, $R^2 = 0.010$; log(daily bouts), $F_{1,79} = 2.341$, $P = 0.130$, $R^2 = 0.029$; Bonferroni correction α-value = 0.006; $N = 81$ bees; forewing length varied by a factor of 1.83 across all foragers).

Results summary

Our results demonstrate that patterns of reward specialization are dramatically different over the short-term versus the long-term. Specifically, while a minority of foragers were lifetime specialists (making >90% of their bouts for a single type of reward), the majority of foragers were daily specialists. Despite making up a majority of workforce on a given day, daily reward specialists contributed significantly less to daily foraging effort than daily reward generalists.

Furthermore, we uncovered significant variation in foraging effort among foragers. Mean daily foraging effort varied nearly 40-fold among foragers, with approximately 17% of bees making 50% of the colony's daily foraging bouts. While high-activity foragers preferred to collect nectar over their lifetime, they were not nectar specialists.

While patterns of daily or lifetime reward specialization were not associated with antennal sensory morphology or other morphological characteristics, bees with more

antennal pore plates foraged for significantly more days in total and made significantly more lifetime pollen bouts.

Patterns of foraging specialization changed significantly with colony age. As the colony aged and the percent of brood decreased, the colony collected proportionally more nectar. Likewise, as the colony aged, fewer foragers specialized daily on pollen and there was a trend for the percentage of foragers that specialized daily on nectar to increase. However, the proportion of daily reward specialists did not change significantly as the colony aged.

DISCUSSION

Previous research has characterized foraging specialization on the two dominant floral rewards, pollen and nectar, at a particular stage in the colony's lifespan, for a subset of foragers, and/or at particular times of day (e.g., for honeybees, stingless bees, and bumblebees[20,37,38,39]). These data snapshots, while informative, offer limited insight into daily and lifetime patterns of reward use for the individual and colony. For bumblebees in particular, the limited evidence to date indicates that individual workers specialize weakly on reward type over their lifetimes (e.g.,[1,36,40]). However, how bees mix collection of pollen and nectar over shorter timescales and how these patterns may change over the life of the colony has so far been unclear. One of the reasons we do not have this information is that it has been logistically difficult to obtain. With the aid of RFID technology, we obtained a complete description of patterns of reward specialization

for all bumblebee foragers in the colony during its growth phase. While logistics dictated the use of a single colony, due to the labour intensive nature of obtaining a colony lifetime record of foraging and forager morphology, future work should also examine between-colony variation.

We found that patterns of specialization did in fact differ greatly depending on the timescale in question. Only a small percentage of foragers specialized on one or the other nutrient over their lifetimes (making >90% of their bouts to a single reward), in accordance with what has been described previously in the literature (e.g.,[1,36]). Yet while most foragers were reward generalists over their lifetimes, greater than half of all foragers specialized on collecting one or the other nutrient on a given day.

Foragers might specialize over short timescales for multiple reasons. For instance, the costs of switching might be particularly high over short timescales. Costs associated with switching between tasks are thought to reduce task performance, thus encouraging a greater degree of task specialization by workers[5,41]. Switching between collecting nectar and pollen over the short term could be costly if, for instance, it took substantial time for a forager to ascertain shifts in colony demand. Why then do foragers switch specializations over longer timescales? Colony nutrient stores and cues released by the brood drive collection of nutrients (e.g.[12,18,19]). Actively foraging workers likely interact sparingly with colony stores and brood. Conversely, workers that are not actively foraging (e.g., during night-time hours) likely interact frequently with colony stores and brood. Consequently, an active forager might respond little to changes in colony demand

over the foraging day. Instead, the forager would rely on an internal motivational state set by prior knowledge of the colony's needs, obtained while that worker was not actively foraging. Upon again attending primarily to tasks within the nest, the forager's internal motivational state would be reset. Such a mechanism of setting and resetting forager motivational state could explain why most bees specialize over short timescales when costs of acquiring information on colony nutrient demand are high, but switch specializations over longer timescales when costs are periodically lower.

Flexible short-term specialization by most foragers could allow the colony to rapidly and efficiently meet shifting nutrient demands. Environmental changes can be more numerous over a long timescale than over a short timescale. For instance, pollen and nectar availability change rapidly over several days or weeks as flowers open, are depleted by visitors, and senesce and as new resource patches become available or decline (see[42]). Although we controlled the foraging environment, the colony itself was a changing environment. In particular, colonies can exhibit strong day-to-day variation in demand for one or the other nutrient (e.g.,[12,18,19]). Accordingly, we found that when brood - which requires copious pollen to develop - was abundant, pollen was the nutrient of choice for short-term specialists. When brood declined, bees were more likely to specialize on nectar collection in the short term.

If specialization led to gains in colony foraging performancewe might expect those gains to be reflected by improvements in individual foraging performance[5]. For instance, specialists might be able to forage more, make quicker bouts, and/or bring back more

nutrients. The data snapshots of prior research in fact suggests that at least some putative nectar and pollen specialists in a colony forage at a higher rate or contribute more to colony foraging effort over a short duration (e.g.,[1,41,45]). The complete description of both reward specialization and foraging effort provided by our RFID system however allowed us to assess this metric of foraging performance for all foragers in a colony across multiple timescales.Interestingly, lifetime specialists made proportionally fewer foraging bouts than lifetime generalists. This result may not be particularly surprising, because lifetime generalists are putatively daily reward specialists. However we were surprised to find that daily reward specialists made proportionally fewer foraging bouts than generalists at the same timescale. One possible explanation is that daily generalists may have been specializing at a different timescale (e.g., shorter or longer than a day) not reported in this study.

Additionally, daily reward specialists might have been better foragers in ways other than foraging effort. For instance, specialists might have been able to collect rewards more quickly or might have collected larger loads, as noted for honeybees[10,43]. These particular gains in foraging performance would have been especially reasonable if reward specialists had been relatively larger. Larger bees are thought to be better foragers because they are able to carry more pollen or nectar per bout and are able to locate and learn about resources more quickly due to their disproportionately larger sensory organs (e.g.,[20,21,22,23]). However, we found no relationship between degree of lifetime reward specialization and body size. The lack of a relationship should not come as a surprise: while some foragers were consistent across days in the degree and type of reward on

which they specialized, the vast majority of foragers instead exhibited substantial variation in both metrics across days. Specifically, most foragers specialized on collecting pollen or nectar on a given day, but individual foragers exhibited a surprising degree of flexibility in how specialized they were on any given day, and body size is fixed in adult insects.

In addition, our results suggest that at least for bumblebees, antennal sensory morphology does not directly govern short or long-term patterns of reward specialization. If lifetime reward preference reflected variation in response thresholds, as commonly presumed for honeybees[4,9], and antennal sensory morphology in turn governed response thresholds[10], short-term reward specialists (lifetime reward generalists) would be expected to exhibit intermediate numbers of pore plates. Yet we did not find that reward specialization was associated with pore plate number. Interestingly, however, we did find that workers with more pore plates made more pollen bouts over their lifetimes. Taken together with[10], this result suggests that pore plates may mediate pollen foraging activity to some degree for eusocial pollinators generally.

Patterns of short-term specialization are one way in which colonies could meet shifting nutrient demands. However, fixed lifetime preferences could also allow colonies to meet a given nutrient demand if lifetime specialists were able to rapidly and flexibly alter their foraging effort (number of bouts). For instance, if demand for pollen exceeded demand for nectar, lifetime specialists on nectar might decrease their foraging effort, while lifetime specialists on pollen might increase their foraging effort. Yet while we found that

foraging effort varied considerably across bees, an individual's foraging effort did not change significantly over lifetime. Instead, greater lifetime foraging effort was associated with a greater number of pore plates (and thus larger body size). This result lends further support to the hypothesis that larger bees with better sensory capabilities also contribute more to nutrient collection[20,21,40]. In fact, a small number of high activity foragers ('elites') contributed disproportionately to foraging effort. Elite foragers appear to be a widespread phenomenon among social insects[29], but have not previously been reported for bumblebees. Our results thus indicate that while individual bumblebee foragers may exhibit tremendous flexibility in patterns of reward specialization over lifetime, foraging effort is not as plastic.

Our results suggest a number of future directions. In this study we forced bees that collected both pollen and nectar to switch between foraging chambers. This design imposed a cost of switching between rewards, which could have contributed to the observed patterns of specialization. Future work should therefore directly investigate whether switching between collection of pollen and nectar is costly (as suggested by[41] for bees foraging from live flowers) and whether imposing greater switching costs might cause a greater degree of short-term reward specialization. Furthermore, although honeybee-collected pollen is regularly used in studies of bee foraging behaviour (e.g.,[36,39,44]) this reward is adulterated with debris and up to 60% sugars[24] and is thus not a realistic substitute for floral pollen that wild bees must collect. While behavioural patterns in our study were robust, the use of honeybee pollen might be misleading, especially where the collection of pollen is concerned. For instance, adulteration with

nectar may have reduced the putative role of pore plates in mediating pollen foraging behaviour in our study. Future work should thus have bees forage for live (unadulterated) floral pollen.

To close, we find that while lifetime reward specialization in bumblebees is rare, short-term specialization on either pollen or nectar appears to be the norm. While reward specialists were not more prolific foragers, we propose that patterns of short-term specialization in particular allow bumblebees colonies to flexibly meet shifts in nutrient demand. Future work will be required to investigate what factors regulate short-term specialization and whether reward specialists at either lifetime or daily timescales also enhance colony performance, for instance, by carrying larger resource loads than generalists at either timescale. Moreover, we found enormous interindividual variation in foraging effort; a small number of foragers contributed disproportionately to foraging effect. Future studies should examine whether these high activity foragers are in fact a unique group of foraging specialists, or whether their heightened foraging activity simply reflects a heightened activity level for all tasks high activity foragers participate in (a 'behavioural syndrome').

ACKNOWLEDGEMENTS

We are grateful to Wulfila Gronenberg for use of his microscopy equipment and to Mary McIntosh for assistance in measuring bee morphological characteristics. This work was

supported by grants from the University of Arizona Graduate & Professional Student Council, the University of Arizona Center for Insect Science, and the National Science Foundation (Award No. IOS-0921280).

AUTHOR CONTRIBUTIONS

ALR & DRP wrote the paper. ALR designed & ran the experiment & the analyses. ALR & SJM designed the RFID data processing software. SJM wrote the RFID data processing software. EHM took bee morphological measures. All authors contributed to revised versions of the manuscript.

DATA ACCESSIBILITY

We will archive data for this project at Dryad on acceptance.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

REFERENCES

1. O'Donnell, S. Reichardt, M. & Foster, R. Individual and colony factors in bumble bee division of labor (*Bombus bifarius nearcticus* Handl; Hymenoptera, Apidae). *Insectes Soc*. **47**, 164-170 (2000).

2. Rueffler, C., Hermisson, J. & Wagner, G. P. Evolution of functional specialization and division of labor. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 1830–1831 (2012).

3. Chittka, L., Thomson, J. D. & Waser, N. M. Flower Constancy, Insect Psychology, and Plant Evolution. *Naturwissenschaften* **86**, 361-377 (1999).

4. Page Jr, R. E., Linksvayer T. A. & Amdam G. V. Social life from solitary regulatory networks: a new paradigm for insect sociality in *Organization of Insect Societies: From Genomes to Socio-complexity* (eds. Gadau, J., Fewell & J. H.) 355-374 (Harvard University Press, 2009).

5. Dornhaus, A. Specialization does not predict individual efficiency in an ant. *PLoS Biol*. http://dx.doi.org/10.1371/journal.pbio.0060285 (2008).

6. Duarte, A., Scholtens, E. & Weissing, F.J. Implications of behavioral architecture for the evolution of self-organized division of labor. *PLoS Comput. Biol.* http://dx.doi.org/10.1371/journal.pcbi.1002430 (2012).

7. Lewis, A. C. Learning and the evolution of resources: pollinators and flower morphology in *Insect learning: ecological and evolutionary perspectives* (eds. Papaj, D.R. & Lewis, A.C.) 219-242 (Chapman & Hall, 1993).

8. Gegear, R. J. & Laverty, T. M. Effect of flower complexity on relearning flower-handling skills in bumble bees. *Can. J. Zool*. 7**3**, 2052-2058 (1995).

9. Page, R. E., Scheiner, R., Erber, J. & Amdam, G. V. The development and evolution of division of labor and foraging specialization in a social insect (*Apis mellifera* L.). *Curr. Top. Dev. Biol*. **74**, 253-286 (2006).
10. Riveros, A. J. & Gronenberg, W. Sensory allometry, foraging task specialization and resource exploitation in honeybees. *Behav. Ecol. Sociobiol*. **64**, 955-966 (2010).

11. Simpson, B. B. & Neff, J. T. Floral rewards: Alternatives to pollen and nectar. *Ann. Missouri Bot. Gard*. **68**, 301-322 (1981).

12. Kitaoka, T. K. & Nieh, J. C. Bumble bee pollen foraging regulation: role of pollen quality, storage levels, and odor. *Behav. Ecol. Sociobiol*. **63**, 625–625 (2009).

13. Zimmerman, M. Optimal foraging: random movement by pollen collecting bumblebees. *Oecologia* **3**, 394-398 (1982).

14. Gonzalez, A. et al. Flower choice by honey bees (*Apis mellifera* L.): sex-phase of flowers and preferences among nectar and pollen foragers. *Oecologia* **101**, 258-264 (1995).

15. Kevan, P. G. & Baker, H. G. Insects as flower visitors and pollinators. *Ann. Rev. Entomol*. **28**, 407-453 (1983).

16. Nicolson, S. W. Bee food: The chemistry and nutritional value of nectar, pollen and mixtures of the two. *Afr. Zool.* **46**, 197e204. http://dx.doi.org/10.1080/15627020.2011.11407495 (2011).

17. Francis, J. S., Muth, F., Papaj, D. R. & Leonard, A. S. Nutritional complexity and the structure of bee foraging bouts. *Behav. Ecol*. **3**, 903-911 (2015).

18. Hellmich, R. L. & Rogthenbuhler, W. C. Relationship between different amounts of brood and the collection and use of pollen by the honey bee (*Apis mellifera*). *Apidologie*, **1**, 13-20 (1986).

19. Plowright, C. M. S., Cohen-Salmon, D., Landry, F. & Simonds, V. Foraging for nectar and pollen on thistle flowers (*Cirsium vulgare*) and artificial flowers: how bumble bees (*Bombus impatiens*) respond to colony requirements. *Behaviour* **136**, 951-963 (1999).

20. Goulson, D. et al. Can alloethism in workers of the bumblebee, *Bombus terrestris*, be explained in terms of foraging efficiency? *Anim. Behav*. **64**, 123-130 (2002).

21. Spaethe, J. & Weidenmuller, A. Size variation and foraging rate in bumblebees (*Bombus terrestris*). *Insectes Soc*. **49**, 142-146 (2002).

22. Spaethe, J. & Chittka, L. Interindividual variation of eye optics and single object resolution in bumblebees. *J. Exp. Biol*. **206**, 3447-3453 (2003).

23. Spaethe, J., Brockmann, A., Halbig, C. & Tautz, J. Size determines antennal sensitivity and behavioral threshold to odors in bumblebee workers. *Naturwissenschaften* **94**, 733-739 (2007).

24. Russell, A. L. & Papaj, D. R. Artificial pollen dispensing flowers and feeders for bee behaviour experiments. *J. Pollinat. Ecol*. **18**, 13-22 (2016).

25. Johnson, S. A. & Cartar, R. V. Wing wear, but not asymmetry in wear, affects load-lifting capability in bumble bees *Bombus impatiens*. *Can. J. Zool*. **92**, 179-184 (2014).

26. Vareschi, E. Odor discrimination by the honeybee: single cell recording and behavior reaction. *Z. Vgl. Physiol*. **75**, 143–173 (1971).

27. Wcislo, W. T. Sensilla numbers and antennal morphology of parasitic and non-parasitic bees (Hymenoptera : Apoidea). *Int. J. Insect Morphol. & Embryol*. **24**, 63-81 (1995).

28. Molet, M., Chittka, L. & Raine, N. E. Potential application of the bumblebee foraging recruitment pheromone for commercial greenhouse pollination. *Apidologie* **40**, 608-616 (2009).

29. Tenczar, P., Lutz, C.C., Rao, V. D., Goldenfeld, N. & Robinson, G. E. Automated monitoring reveals extreme interindividual variation and plasticity in honeybee foraging activity levels. *Anim. Behav*. **95**, 41-48 (2014).

30. R Development Core Team. R: a language and environment for statistical computing. Vienna (Austria): R Foundation for Statistical Computing (2010).

31. Beals, M., Gross, L. & Harrell, S. (n.d.). What is a learning curve? Diversity indices: Shannon's H and E http://www.tiem.utk.edu/~gross/bioed/bealsmodules/shannonDI.html (accessed Oct 2016).

32. Gegear, R. J. & Thomson, J. D. Does the flower constancy of bumble bees reflect foraging economics? *Ethol*. **110**, 793-805 (2004).

33. Cheng, R. Tools for mapping multiple complex traits. R package version 0.1-4 [cited 2016 Oct 8]. https://cran.r-project.org/web/packages/qtlmt/qtlmt.pdf (2015).

34. Fox, J. & Weisberg, S. An {R} Companion to Applied Regression, Second Edition. Thousand Oaks CA: Sage. http://socserv.socsci.mcmaster.ca/jfox/Books/Companion (2011).

35. Kuznetsova, A. Brockhoff, P.B. & Christensen, R.H.B. lmerTest: Tests in Linear Mixed Effects Models. R package version 2.0-32 [cited 2016 Oct 8]. https://cran.r-project.org/web/packages/qtlmt/qtlmt.pdf (2016).

36. Hagbery, J. & Nieh, J. C. Individual lifetime pollen and nectar foraging preferences in bumble bees. *Naturwissenschaften*. **99**, 821-32 (2012).

37. Ribbands C. R. Division of labour in the honeybee community. *Proc. R. Soc. Lond. B Biol. Sci*. **140**, 32-43 (1952).

38. Biesmeijer, J. C. & Tóth, E. Individual foraging, activity level and longevity in the stingless bee *Melipona beecheii* in Costa Rica (Hymenoptera, Apidae, Meliponinae). *Insectes Soc*. **45**, 427-443 (1998).

39. Konzmann, S. & Lunau, K. Divergent rules for pollen and nectar foraging bumblebees--a laboratory study with artificial flowers offering diluted nectar substitute and pollen surrogate. *PLoS One* **17**, e91900 http://dx.doi.org/10.1371/journal.pone.0091900 (2014).

40. Free, J. B. The division of labour within bumblebee colonies. *Insectes Soc*. **3**, 195-212 (1955).

41. Cartar, R. V. Adjustment of foraging effort and task switching in energy-manipulated wild bumblebee colonies. *Anim. Behav*. **44**, 75-87 (1992).

42. Goulson, D. Foraging strategies of insects for gathering nectar and pollen, and implications for plant ecology and evolution. *Perspect. Plant Ecol. Evol. Syst*. **2**, 185-209 (1999).

43. Pankiw, T., Tarpy, D. R. & Page, R. E. Genotype and rearing environment affect honeybee perception and foraging behaviour. *Anim. Behav*. **64**, 663-672 (2002).

44. Nicholls, E. & Hempel de Ibarra, N. Bees associate colour cues with differences in pollen rewards. *J. Exp. Biol*. **217**, 2783-2788. http://dx.doi.org/10.1242/jeb.106120 (2014).

45. Hofstede, F. E. & Sommeijer, M. J. Effect of food availability on individual foraging specialisation in the stingless bee *Plebeia tobagoensis* (Hymenoptera, Meliponini). Apidologie, **37**, 387-397 (2006).

FIGURE LEGENDS

Figure 1. Flowering plant species offer pollen and nectar rewards in all combinations: four possibilities are presented here. (a) *Begonia odorata* offers only pollen. (b) *Asclepias subulata* offers only nectar; pollen is held in specialized protective pollinia. (c) *Hedysarum boreale* offers pollen and nectar rewards simultaneously; a pollinator collects both. (d) Young (pinkish) *Mertensia paniculata* flowers contain only pollen while old (blue) flowers produce nectar (Morris 1996); a pollinator collects nectar from an old flower. Photographs: (a,c,d): Avery Russell; (b): Stan Shebs, licensed by CC BY-SA 3.0.

Figure 2. Elements of the experimental foraging design. (a) Forward view of the foraging arena with each foraging chamber in view. Four of the 6 custom-built copper feeder-holders can be seen. The red-dashed line indicates the location of the plywood wall separating the arena into two foraging chambers. The white triangles surround the chamber entrances. (b) Paired RFID reader holder and chamber entrance. (c) Paired RFID readers mounted on holder, joined to tunnel. (d) Paired RFID readers and forking tunnel setup. Purple ring attaches to a tunnel (not shown) that in turn connects to the colony box.

Figure 3. Representative *Bombus impatiens* forewing and proboscis scanned at 2400dpi and portion of an antenna's nail polish cast. (a) Right forewing and (b) proboscis. White arrows indicate start and end points for length measures made in ImageJ. (c) Nail polish cast of the 7^{th} antennal segment showing the boundary between the zone with pore plates

(bottom) and the zone with only hair plates (top). White arrows indicate several pore plates at the boundary.

Figure 4. Patterns of reward specialization and of foraging effort for bees with a given degree of specialization, over both short and long timescales. Percentage of foragers with a given (a) lifetime or (b) mean daily nectar foraging preference (+SE). Percentage of (c) colony lifetime or (d) mean daily foraging bouts made by foragers with a given (c) lifetime or (d) mean daily nectar foraging preference (+SE). Bin width = 10%; range = 0.0 - 100.0 % nectar foraging preference; N = 97 bees and 43 days. (e) A 2-week foraging period for exemplars of the three major types of reward specialists: a bee that specialized on nectar collection over its lifetime (top trace - blue line); a bee that specialized on pollen collection over its lifetime (bottom trace - yellow line); and a bee that specialized on either nutrient over the short-term (middle trace - black line). (f) Jacob's Constancy Index (CI) for forager lifetime: bees that were more inconstant (systematically alternated between rewards) have values closer to -1; bees that made random transitions between rewards have values closer to 0; bees that foraged in runs for one or the other reward have values closer to 1; bin width = 0.1; N = 68 bees. (g) Shannon Diversity Index (H) describing variation in daily degree of reward specialization over forager lifetime: bees with less flexibility in their reward specialization across days over lifetime have values closer to 0; bin width = 1; N = 103 bees.

Figure 5. Percentage of foragers that make a given daily mean number of foraging bouts and standard error (S.E.) around the means. (a,b) Nectar and pollen bouts summed (c,d)

nectar (blue bars) and pollen (yellow bars) bouts displayed separately. Bin width for means = 5 bouts; range: all bouts, 1.0 - 38.4 bouts per day; nectar bouts, 0.0 - 35.8; pollen bouts, 0.0 - 15.85. Bin width for variances = 1 bout; range: all bouts, 0.2 - 8.7; nectar bouts, 0.1 - 6.9; pollen bouts, 0.1 - 5.6. N = 97 bees and 43 days.

Figure 6. Log of the mean daily number of foraging bouts made by foragers with a given lifetime nectar collection preference. (a) Lifetime nectar foraging preference plotted against nectar (blue points) and pollen (yellow points) bouts, displayed separately, (b) nectar bouts plotted against pollen bouts; slope of 1 indicated by red dashed line. N = 89 bees. Plots with trend lines indicate a statistically significant relationship ($p < 0.05$) according to a Type II ANOVA and an LM, respectively.

Figure 7. Effects of colony age on its foraging behaviour and on its reproduction. (a) Percent of the colony surface composed of brood cells, (b) colony nectar collection preference, (c) number of active foragers, (d) mean number of bouts per bee, (e) percentage of bees that specialized on nectar collection, (f) percentage of bees that specialized on pollen collection, and (g) percentage of bees that specialized on either nutrient, for each day of the experiment. N = 89 bees. N = 33 days for (a-d) and 43 days for (e-g). Plots with trend lines indicate a statistically significant relationship ($p < 0.05$) according to LMs; although the analysis for (c) was performed via a quadratic model, the quadratic fit for the untransformed data is shown here.

Figure 8. The relationship between forager antennal sensory morphology and lifetime foraging patterns. Lifetime number of (a) active foraging days, (b) nectar and pollen bouts summed, (c,d) pollen and nectar bouts displayed separately, and (e) the lifetime preference of foragers to collect nectar. The number of pore plates is reported for the 7^{th} antennal segment of each forager. $N = 81$ bees. Plots with trend lines indicate a statistically significant relationship ($p < 0.05$) according to LMs.

FIGURES

Figure 1

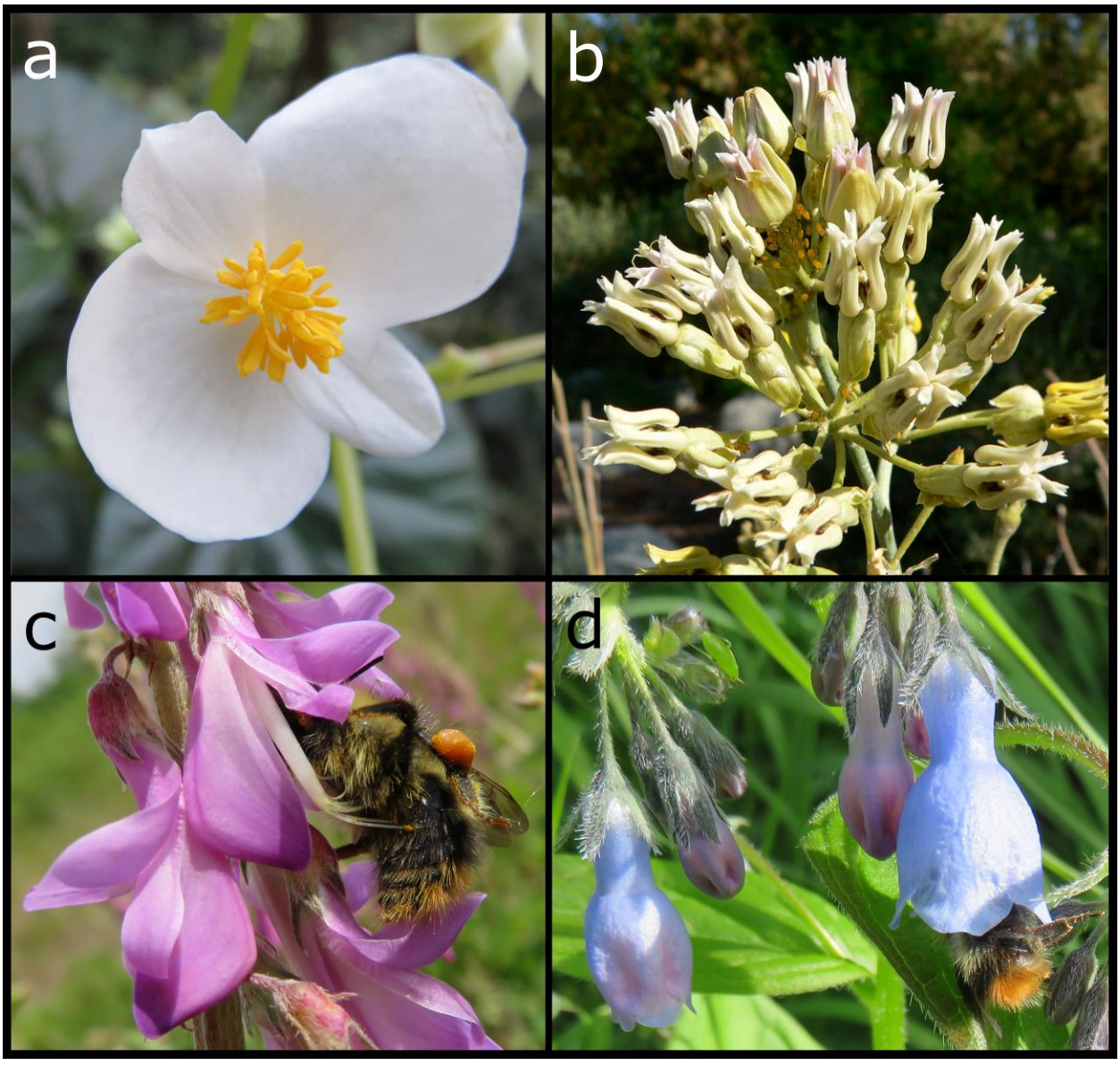


Figure 2

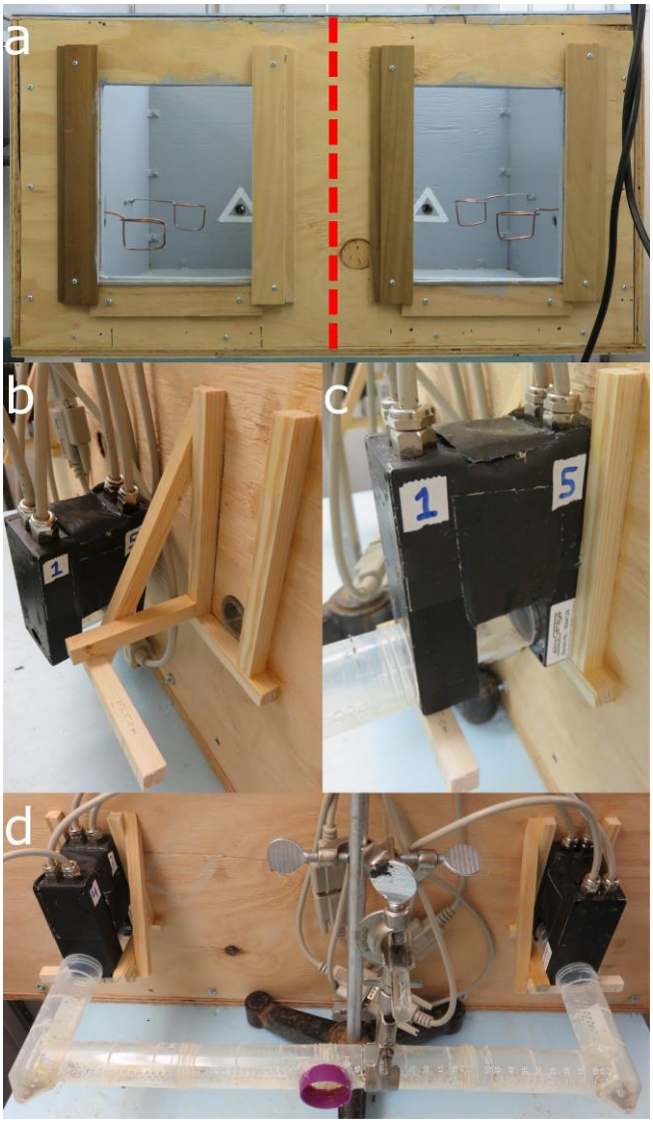


Figure 3

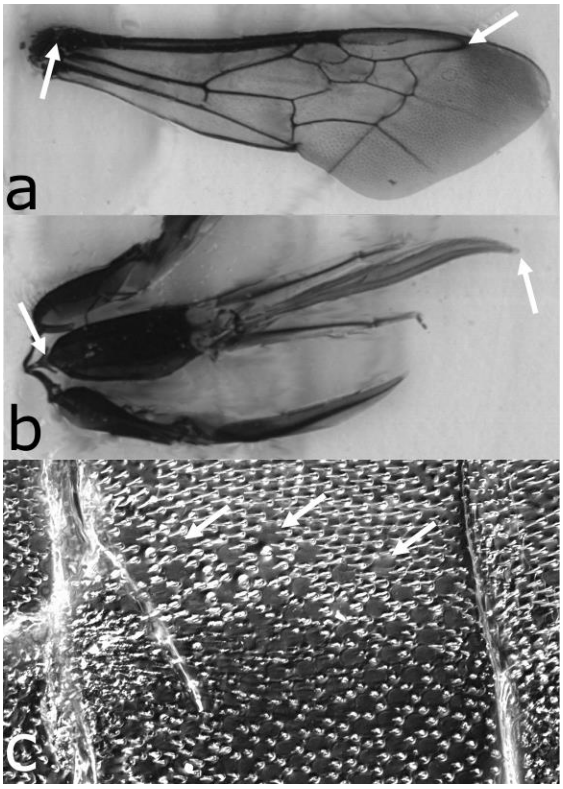


Figure 4

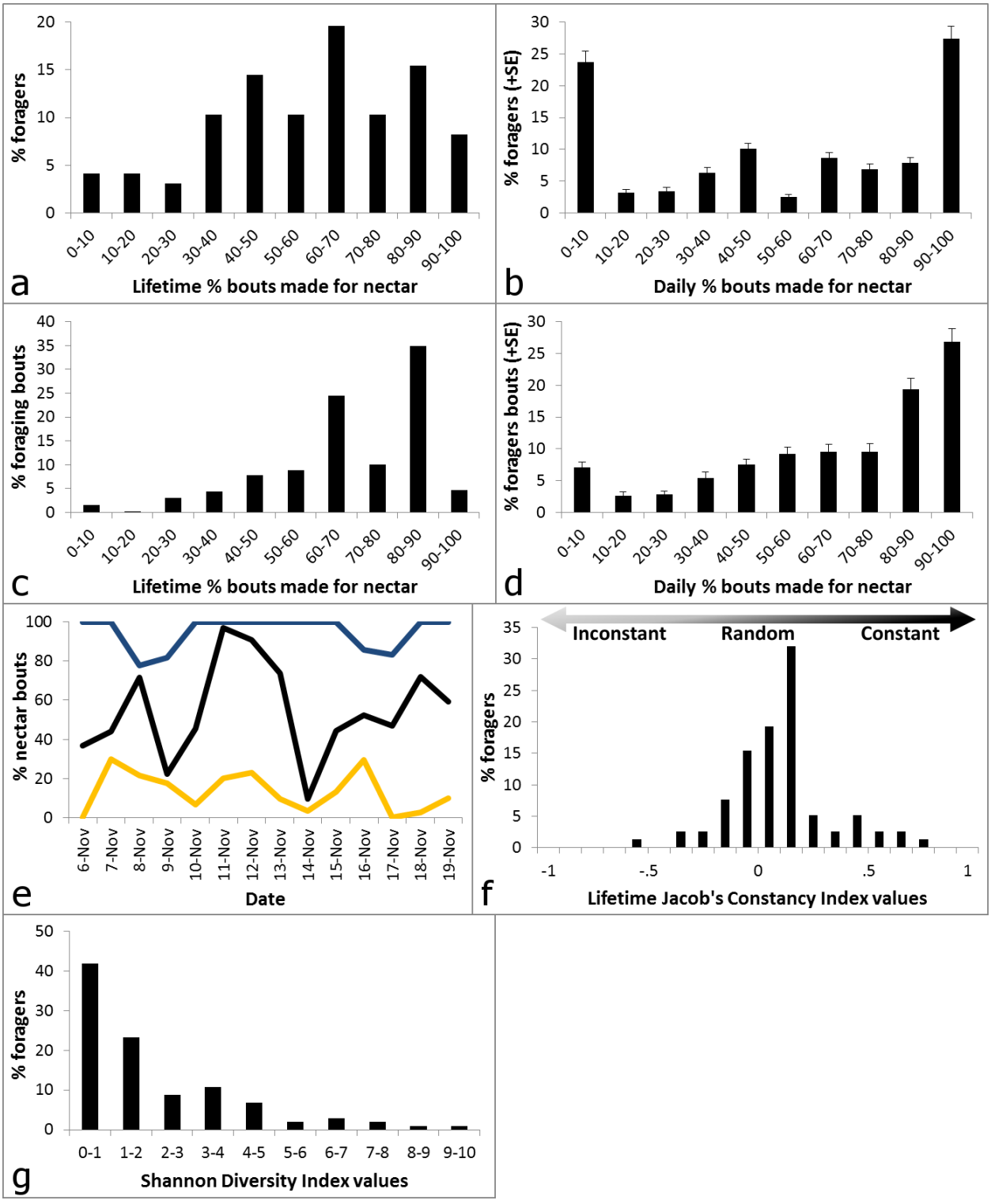

% foragers
0-10
10-20
20-30
30-40
40-50
50-60
60-70
70-80
80-90
90-100
a
Lifetime % bouts made for nectar
% foragers (+SE)
b
Daily % bouts made for nectar
% foraging bouts
c
Lifetime % bouts made for nectar
% foragers bouts (+SE)
d
Daily % bouts made for nectar
% nectar bouts
6-Nov
7-Nov
8-Nov
9-Nov
10-Nov
11-Nov
12-Nov
13-Nov
14-Nov
15-Nov
16-Nov
17-Nov
18-Nov
19-Nov
e
Date
Inconstant
Random
Constant
% foragers
f
Lifetime Jacob's Constancy Index values
% foragers
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
g
Shannon Diversity Index values

Figure 5

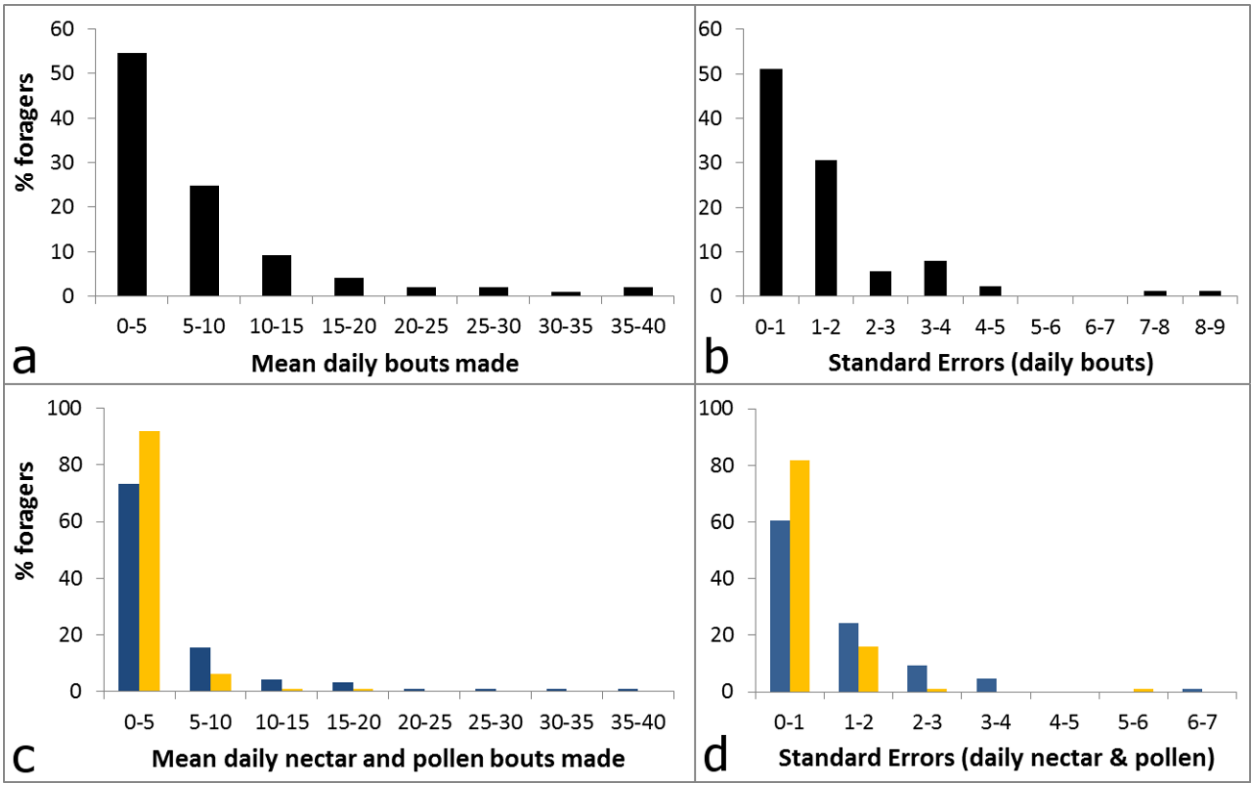


Figure 6

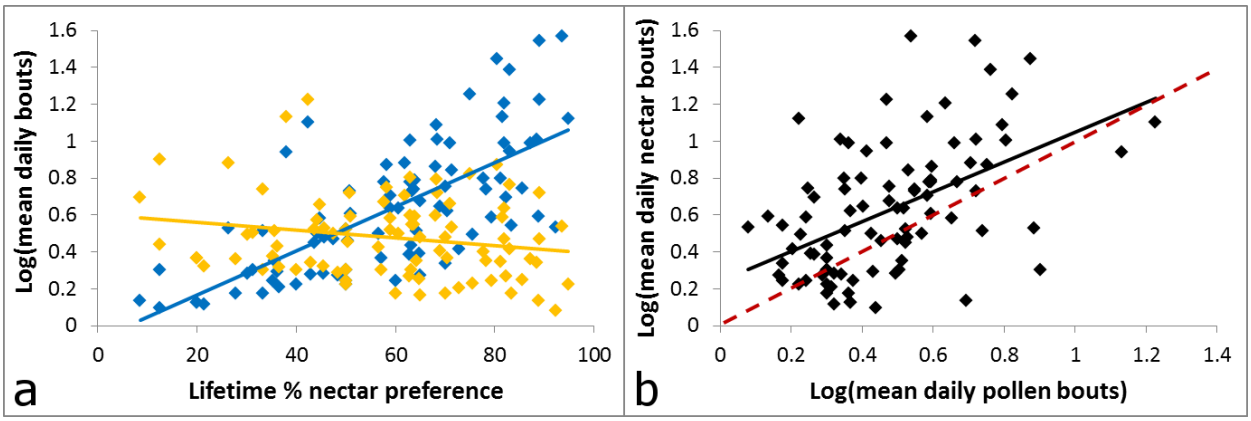


Figure 7

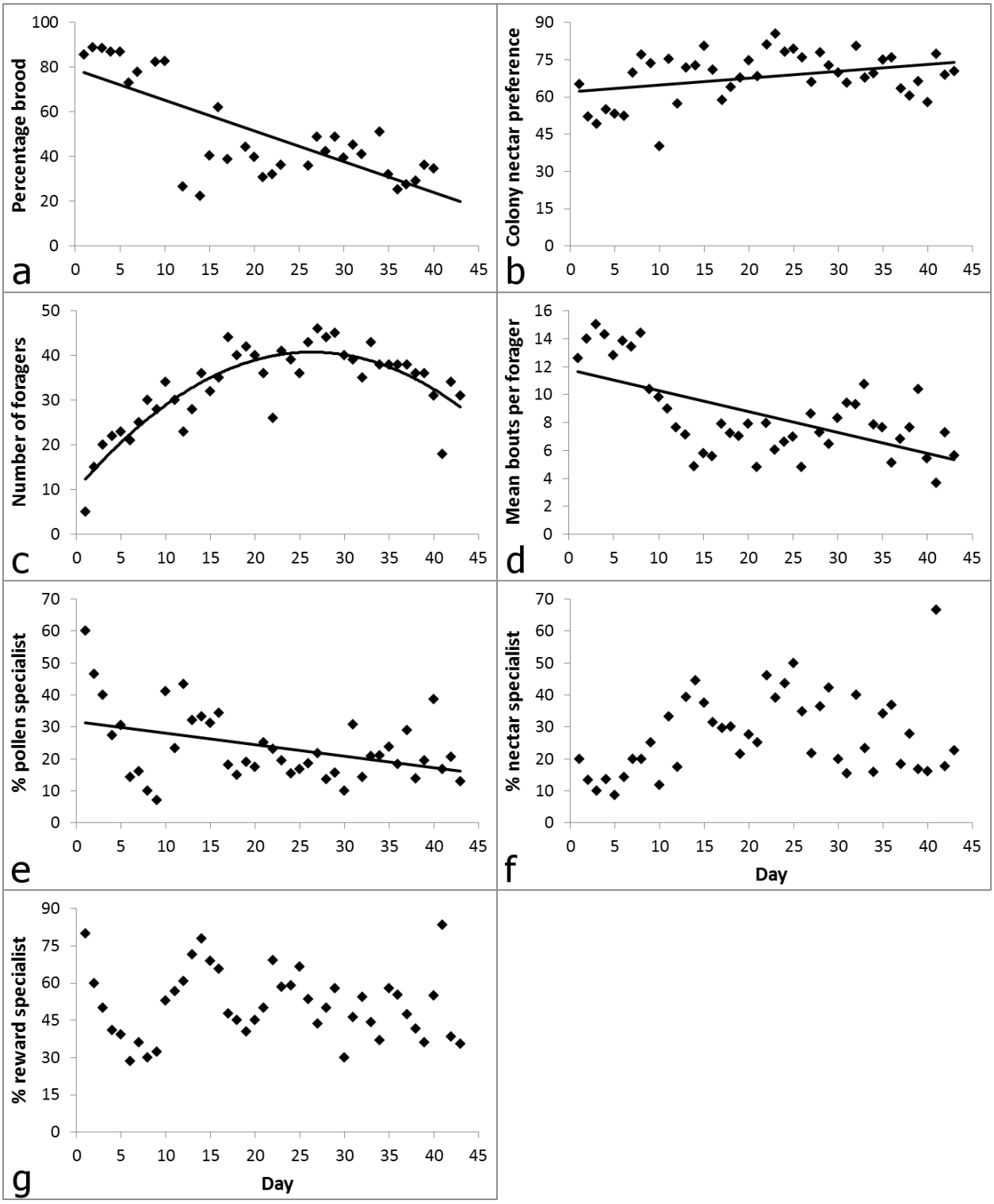
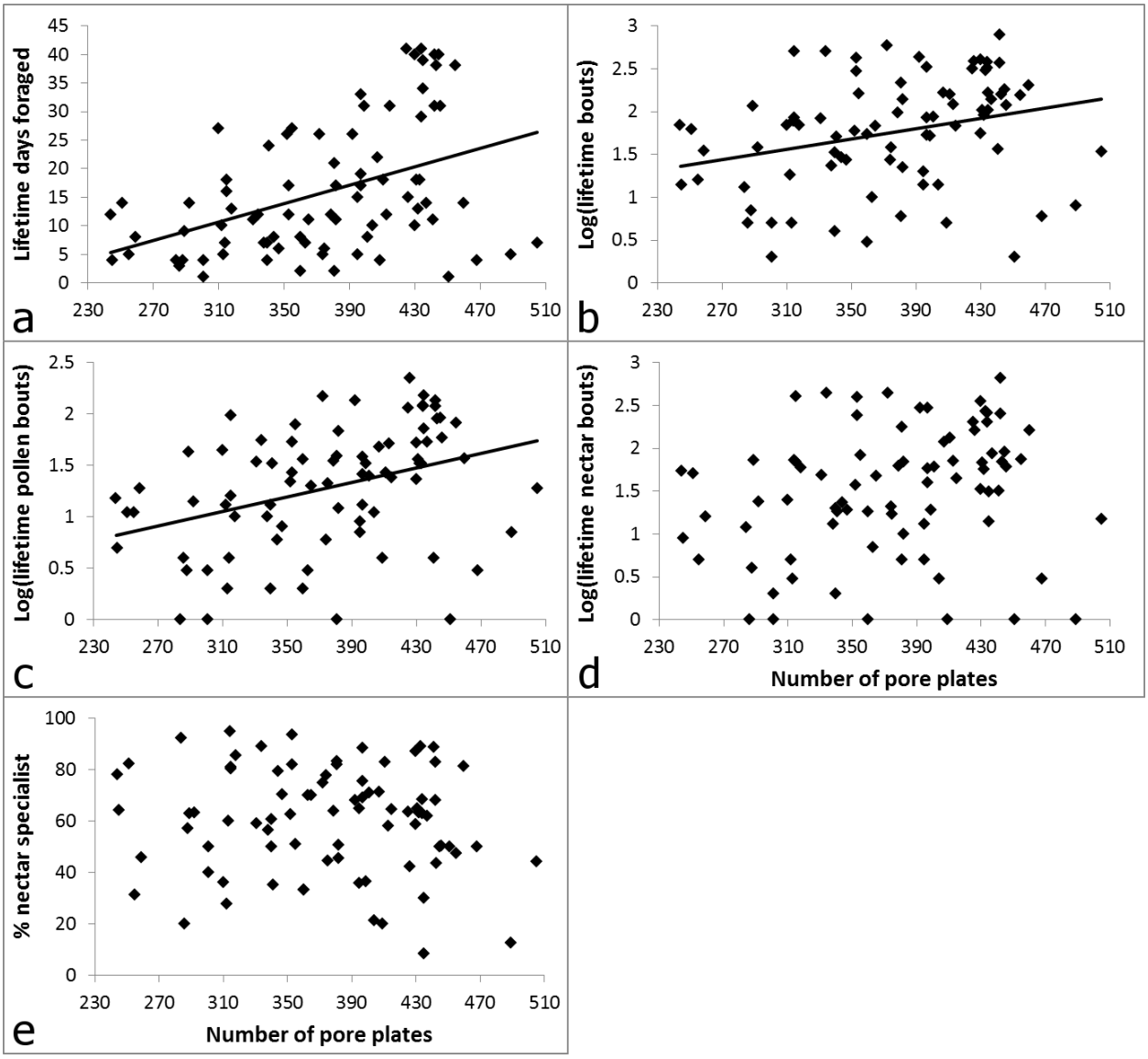

Percentage brood
a
Colony nectar preference
b
Number of foragers
c
Mean bouts per forager
d
% pollen specialist
e
% nectar specialist
f
Day
% reward specialist
g
Day

Figure 8


Lifetime days foraged
45
40
35
30
25
20
15
10
5
0
230 270 310 350 390 430 470 510
a
Log(lifetime bouts)
3
2.5
2
1.5
1
0.5
0
230 270 310 350 390 430 470 510
b
Log(lifetime pollen bouts)
2.5
2
1.5
1
0.5
0
230 270 310 350 390 430 470 510
c
Log(lifetime nectar bouts)
3
2.5
2
1.5
1
0.5
0
230 270 310 350 390 430 470 510
Number of pore plates
d
% nectar specialist
100
80
60
40
20
0
230 270 310 350 390 430 470 510
Number of pore plates
e

SUPPLEMENTARY MATERIALS

No systematic asymmetry between forewings and between antennae

We found little difference between forewings and between antennae for each bee and no systematic asymmetry (paired *t*-tests: pore plate number, $t_{68} = 0.487$, $P = 0.628$; forewing length, $t_{108} = 0.124$, $P = 0.902$; mean percent similarity ± SE: pore plate number, 93.65 ± 0.83, range = 219-505, $N = 69$ bees; forewing length, 97.60 ± 0.30, $N = 109$ bees).

Table S1: Correlation (*r*) between morphological measures

	Forewing length	**Head width**	**Proboscis length**	**Pore plate number**	**Antennal length**	**7th ant. segment width**	**7th ant. segment length**	**Pore plate density**
Forewing length		0.90	0.84	0.76	0.92	0.85	0.82	-0.20
Head width	0.90		0.74	0.66	0.97	0.89	0.88	-0.35
Proboscis length	0.84	0.74		0.66	0.75	0.69	0.64	-0.10
Pore plate number	0.76	0.66	0.66		0.73	0.70	0.65	0.33
Antennal length	0.92	0.97	0.75	0.73		0.92	0.91	-0.30
7th ant. seg. width	0.85	0.89	0.69	0.70	0.92		0.86	-0.38

7th ant seg. length	0.82	0.88	0.64	0.65	0.91	0.86		-0.41
Pore plate density	-0.20	-0.35	-0.10	0.33	-0.30	-0.38	-0.41	

All morphological measures, except for pore plate density, were strongly correlated with one another. Eight bees were discarded due to missing values in one or several morphological categories; N = 101 bees.

Table S2: Correlation (*r*) between lifetime behavioural measures

	Lifetime bouts	**Lifetime nectar bouts**	**Lifetime pollen bouts**	**Total days foraged**	**Lifetime nectar preference**
Lifetime bouts		0.97	0.75	0.59	0.28
Lifetime nectar bouts	0.97		0.56	0.47	0.38
Lifetime pollen bouts	0.75	0.56		0.69	-0.1
Total days foraged	0.59	0.47	0.69		0.01
Lifetime nectar preference	0.28	0.38	-0.1	0.01	

All lifetime behavioural measures were strongly correlated with one another, except for lifetime nectar preference with total days foraged, or with lifetime pollen bouts. Eleven bees that did not make enough bouts to calculate lifetime nectar preference were discarded. N = 98 bees.

Figure S1

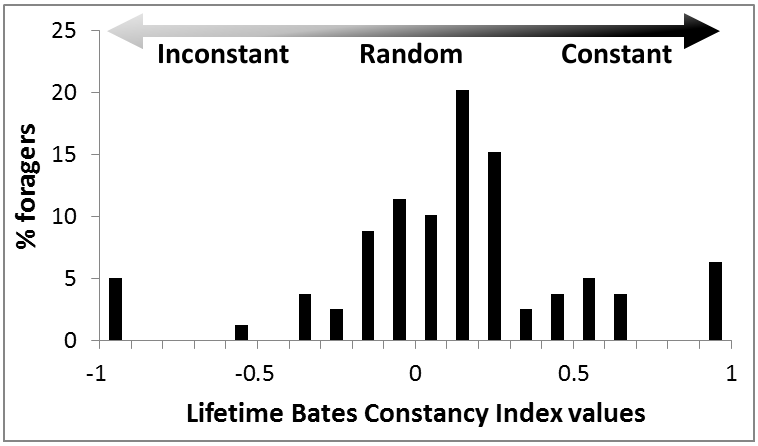


Figure S1. Bates Constancy Index (CI) for forager lifetime: bees that were more inconstant (systematically alternated between rewards) have values closer to -1; bees that made random transitions between rewards have values closer to 0; bees that foraged in runs for one or the other reward have values closer to 1; bin width = 0.1; mean Bates CI ± SE = 0.12 ± 0.05; *N* = 79 bees.

Daily cycle of foraging activity

Daily foraging activity and the number of active foragers increased rapidly with onset of light, peaking 2-3 hours before mid-day and falling off ~1-2 hours before the lights shut off (Fig. S2). Activity preceded light onset by approximately 1 hour and dropped off steeply when the lights shut off 12 hours later (Fig. S2).

Figure S2

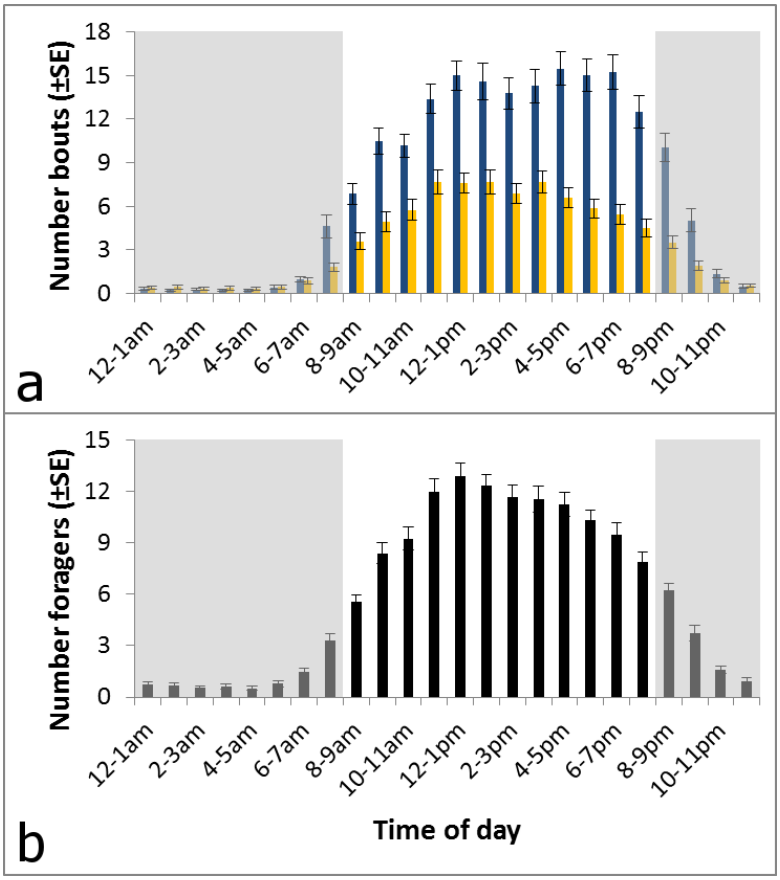


Figure S2. Mean daily foraging activity (+SE) and foraging bouts (±SE) for nectar and pollen. Nectar and pollen bouts indicated by blue and yellow bars, respectively. Grey shading indicates night-time hours. Bin width = 1 hour; *N* = 43 days.

APPENDIX B

BEES LEARN PREFERENCES FOR PLANT SPECIES THAT OFFER ONLY POLLEN AS A FLORAL REWARD

FULL TITLE: Bees learn preferences for plant species that offer only pollen as a floral reward

RUNNING TITLE: Learned preferences for pollen-only plant species

Avery L. Russell[a*], Rebekah E. Golden[b], Anne S. Leonard[c], and Daniel R. Papaj[b]

[a] Entomology and Insect Science Graduate Interdisciplinary Program, University of Arizona, Tucson, AZ, 85721. USA

[b] Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, 85721. USA

[c] Department of Biology, University of Nevada, Reno, NV, 89557. USA

*Corresponding author

This manuscript has been published:

Russell, A.L., Golden, R.E., Leonard, A.S. and Papaj, D.R. 2016. Bees learn preferences for plant species that offer only pollen as a reward. *Behavioral Ecology. 27: 731-740, doi: 10.1093/beheco/arv213*

ABSTRACT

The astonishing diversity of floral form in angiosperm plants is driven in large part by preferences of pollinators for various floral traits, including learned preferences. Remarkably, almost all of a vast literature on learning and memory in pollinators relates to nectar as a reward, even though bees and many flies, beetles, and butterflies must collect pollen. In this study, we asked if bees formed preferences for plant species from which pollen had been collected successfully. Using absolute conditioning, we gave pollen-foraging bees experience with plant species that offered only pollen rewards. Naïve bees generally showed modest preferences, whereas experienced bees adopted strong preferences for those species over alternative species not previously experienced. Learned preferences were retained for at least 24 hours, consistent with preferences learned with nectar rewards. These experience-mediated changes in preference raised the possibility that bees formed associations between particular floral features and pollen rewards. We therefore asked if learned preferences required that bees successfully collect pollen. Using differential conditioning, we determined that learned preferences were strongly influenced by receipt of a pollen reward. In a final experiment, we characterized the importance of two floral features, the corolla and the anther, in the expression of learned preferences. While experience altered responses to both floral parts, responses to anthers were influenced more strongly. We discuss recent evidence in the literature for associative learning with pollen rewards, and propose that learned preferences in the context of pollen collection have played an important role in floral display evolution.

KEY WORDS

Key words: learning, bumblebee, preference, pollen, floral rewards, floral display

LAY SUMMARY

The amazing diversity of flowers is shaped by the preferences of pollinators foraging for flowers. Although bees must collect both pollen and nectar, learned preferences for flowers have only been demonstrated for nectar foragers. We present evidence for learned preferences by pollen foragers and show that experience specifically affects their responses to the pollen-bearing anthers. We conclude that pollen foraging behavior must be considered when seeking to account for similarities and differences in flowers of different species.

INTRODUCTION

The evolution of biotic resources can be profoundly influenced by the behavior of animals using those resources (Mallet & Joron 1999; Bond & Kamil 2005; Grabowski & 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 both in floral signals and floral rewards.

Although learned preferences have been shown in all kinds of pollinators from vertebrates to insects (e.g. Fukushi 1989; Lunau 1992; Hurly & Healy 2002; Kelber & Pfaf 1997; Weiss 1997; Chittka & 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 & Masek 2014). Bees learn an impressive assortment of floral properties, including colors, iridescence, patterns, polarization, scents; petal microtexture and even electrical fields (Giger & Srinivasan 1995; Schiestl & Gumbert 2000; Whitney et al. 2009ab; Johnson 2013; Clarke et al. 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 & Thomson 2005; Leonard and Papaj 2011; Leonard et al. 2011a; Hopkins & 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 & 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, heat and shelter (Simpson & Neff 1981; Fenster et al. 2004; Seymour & Mathews 2006; Luo et al. 2010; Waser & Ollerton 2006). In particular, nectar is just one of two dominant rewards offered by plants, the other being pollen (Simpson & Neff 1981; Kevan & Baker 1983; Morse 1982; Kitaoka & Nieh 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 (c.f. Kevan & Baker 1983; Nicolson & 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; SL Buchmann pers. comm.).

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; Fig. 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 one or 24 hours 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 two pollen-only species.

METHODS

Subjects

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

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

We used freshly clipped flowers from four *Solanum tridynamum*, seven *Solanum elaeagnifolium*, and 50 *Exacum affine* (a mix of Champion Blue, Little Champ Blue, and Royal Blue) in experiments. All three 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. elaeagnifolium* 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 14h:10h 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 (LxWxH, 82cm x 60cm x 60cm) 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 naïve 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 2M 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 (Fig. 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 pre-determined 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 five minutes. However, virtually all bees reached the maximum number of allowed visits during training and testing. Upon completion of an assay, a bee was euthanized.

Video for all tests was captured at 30fps 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 Lavaliere 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 two 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 three 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 upon 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 seven colonies.

We assayed initial species preference by presenting one set of bees with arrays consisting of species pairs: either ten *S. tridynamum* and ten *S. elaeagnifolium,* or ten *S. tridynamum* and ten *E. affine*. We did not use the third possible species pair (*S. elaeagnifolium* versus *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 one bee which completed only nine.

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, one set of trained bees were allowed to enter the testing arena and forage 20-40 minutes 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 hours after training.

Experiment 2: Differential Conditioning

Here we sought to determine if the changes in preference observed in Experiment 1 was due at least in part on receipt of a pollen reward. This experiment used 60 bees from three 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 poricidal anther with a clean toothpick and allowed to dry for 5 minutes. 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 minutes.

We first assayed preference by presenting one set of flower-naïve bees using an array composed of four *S. tridynamum* and four *E. affine* flowers, all of which were unrewarding. Flowers were arranged in a 3x3 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 2x2 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 six times (three presentations of each array). Switching arrays took approximately 20 seconds each time. Within a trial we used the same arrays in each of the presentations, but after a bee had made two 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 two 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 3x3 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 three colonies.

Each of four types of targets used in this experiment was constructed from two freshly clipped flowers (Fig 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 (Fig. 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, two mosaic types and two 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 one set of bees with 4 x 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 x 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 x 4 array consisting of equal numbers of each sham and mosaic targets 20-40 minutes 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). 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 naïve 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 chisquare (χ^2) 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. elaeagnifolium*; *S. tridynamum* or *E. affine*) and 'treatment' (Control, *S. tridynamum,* or *S. elaeagnifolium*; 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 two 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 & Toomet 2011, Croissant 2012).

RESULTS

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

In the absolute conditioning assay (Experiment 1), bees given experience foraging for pollen on *S. tridynamum* or *S. elaeagnifolium* and then tested in an *S. tridynamum* / *S. elaeagnifolium* array after 20-40 minutes (short term retention test) or after 1 day (long term retention test) preferred the experienced flower type (Fig. 3a; GLMER overall effect for the short term retention test: Type II Wald χ^2 tests for experience × species choice: $\chi^2 = 30.688$, df = 2, $P < 0.0001$; Fig. 3b; GLMER overall effect for the long term retention test: Type II Wald χ^2 tests for experience × 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 20-40 minutes (short term retention test) or after 1 day (long term retention test) also preferred the experienced flower type (Fig. 3c; GLMER overall effect for the short term retention test: Type II Wald χ^2 tests for

experience × species choice: $\chi^2 = 30.154$, df = 2, P < 0.0001; Fig. 3d; GLMER overall effect for the long term retention test: Type II Wald χ^2 tests for experience × species choice: $\chi^2 = 28.607$, df = 2, P < 0.0001).

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

For this analysis we pooled data for naïve bees from the same species pairings within Experiment 1. This was done so that we could achieve a large enough sample size to analyze differences in naïve 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* vs *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 *S. elaeagnifolium* when foraging in the rewarding *S. tridynamum* / *S. elaeagnifolium* array (Experiment 1) (% bees that made their first landing on *S. tridynamum*: 40.0%; binomial probability: P > 0.097, *N*=25). Additionally, naïve 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* x *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 minutes 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 (Fig. 3e; GLMER overall effect: Type II Wald χ^2 tests for experience × species choice: $\chi^2 = 14.659$, 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 minutes preferred the anthers of the experienced flower species (Fig. 4a; GLMER overall effect: Type II Wald χ^2 tests for experience × anther species choice: $\chi^2 = 49.512$, 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 naïve bees (Fig. 4b; GLMER overall effect: Type II Wald χ^2 tests for experience × corolla species choice: $\chi^2 = 15.441$, df = 2, $P < 0.0005$).

Anther and corolla generally did not interact to affect preference

Across the three 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 x anther species: $t = -16.388$, $P < 0.0001$; flower choice x corolla species: $t = -3.242$, $P < 0.002$; flower choice x anther species:corolla species: coefficient estimate = 0.224, $t = 0.669$, $P = 0.503$). Nevertheless, there was one 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 naïve 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 & 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 & 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 conditions is relatively uncommon (e.g. Schemske & Bradshaw 1999; Cane 2011; Dobson 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 one 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 (Fig. S2). Similarly, the floral display typically consists of multi-

component 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 and colleagues 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 Grüter et al. 2008; Arenas & Farina 2012; Nicholls & 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 & Lane 1985; Dafni & 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, since 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 1989; Burkart et al. 2013; A. Russell, A. Leonard & D. Papaj unpubl. 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. tridynamum* (A. Russell and D. Papaj, unpubl. data).

What are the implications of the pollen-foraging preferences characterized here for the evolution of floral morphology and chemistry? While 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 & Vallejo Marin 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 (Fig. 1). While 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 1988; Buchmann & Cane 1989), their behavior has likely been a major force in convergence and divergence relating to this floral "syndrome" (Buchmann 1983; De Luca & Vallejo-Marin 2013). For example, bees' preferences for strong center-surround color contrast (Lunau 1991; Lunau 1994; Lunau 2007) 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 & Lane 1985; Dafni & Kevan 1996; Hansen et al. 2012), and also to direct foragers to the anthers. At short range, anther cues may become more detectable and since 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 (Fig. 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).

DATA ACCESSIBILITY

We will archive data for this project at Dryad upon acceptance.

FUNDING

This work was supported by the Graduate & Professional Student Council and the National Science Foundation (IOS-1257762 to A.S.L. & D.R.P.).

ACKNOWLEDGEMENTS

We are grateful to Jake Francis, Felicity Muth, and two anonymous reviewers for their insightful comments, Carla Essenberg, Madhu Viswanathan, and Kenneth Train for aid with statistical analyses, to Abreeza Zegeer for greenhouse care, and to China Rae Newman, Kevin Mauerman, Sarah White, and Tara Hall for assistance in running experimental trials.

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, URL https://CRAN.R-project.org/package=lme4.

Bergström G, Dobson HEM, Groth I. 1995. Spatial fragrance patterns within the flowers of *Ranunculus acris (Ranunculaceae).* Pl Syst Evol. 195: 221-242.

Bond AB, Kamil AC. 2005. Spatial heterogeneity, predator cognition, and the evolution of color polymorphism in virtual prey. PNAS. 103: 3214-3219.

Buchmann SL, Cane JH. 1989. Bees assess pollen returns while sonicating *Solanum* flowers. Oecologia. 81: 289-294.

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.

Burkart A, Schlindwin C, Lunau K. 2013. Assessment of pollen reward and pollen availability in *Solanum stramoniifolium* and *Solanum paniculatum* 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. 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 multinomial logit model in R: the package mlogit. R package version 0.2-3, URL 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.

De Luca PA, Vallejo-Marín M. 2013. What's the "buzz" about? The ecology and evolutionary significance of buzz-pollination. Curr Opin Plant Biol. 16: 429-435.

Dobson HEM, Bergström G, Groth I. 1990. Differences in fragrance chemistry between flower parts of *Rosa rugosa* Thunb. (*Rosaceae*). Israel J Bot. 39: 143-156.

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

Dobson HEM, Danielson EM, Van Wesep ID. 1999. Pollen odor chemicals as modulators of bumble bee foraging on *Rosa rugosa* Thunb. (*Rosaceae*). Plant Species Biol. 14: 153-166.

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 bicornis* (*Megachilidae*)? Apidologie 43: 698-714.

Faegri K. 1986. The solanoid flower. Trans Bot Soc Edinburgh. 45: 51-59.

Fenster CB, Armbruster WS, Wilson P, Dudash MR, Thomson JD. 2004. Pollination syndromes and floral specialization. Annu. Rev. Ecol. Evol. Syst. 35: 375-403.

Foster JJ, Sharkey CR, Gaworska VA, Roberts NW, Whitney HM, Partridge JC. 2014. Bumblebees learn polarization patterns. Curr Biol. 24: 1415-1420.

Fox J. 2015. Applied regression analysis and generalized linear models, 3rd ed. USA: 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: 791-795.

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, Chapmann 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.

Grabowski 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 preferences 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 R Soc B. 279:634-639.

Henningsen A, Toomet O. 2011. maxLik: A package for maximum likelihood estimation in R. Computational Statistics 26: 443-458. DOI 10.1007/s00180-010-0217-1.

Hopkins R, Rausher MD. 2012. Pollinator-mediated selection on flower color allele drives reinforcement. Science. 335: 1090-1092.

Hothorn T, Bretz F, Westfall P, Heiberger RM, Scheutzenmeister A, Scheibe S. 2015. Simultaneous inference in general parametric models. R package version 1.4-1, URL http://CRAN.R-project.org/package=multcomp.

Hurly 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 R Soc B. 281: 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. 1985. Flower petal microtexture is a tactile cue for bees. Proc Natl Acad Sci. 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, 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.

Leonard AS, Dornhaus 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, Dornhaus A, Papaj DR. 2011b. Why are floral signals complex? An outline of functional hypotheses. In: S. Patiny, editor. Evolution of plant-pollinator relationships. Cambridge U Press. P. 279-300.

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.

Lunau K, Wacht S. 1994. Optical releasers of the innate proboscis extension in the hoverfly *Eristalis tenax* L. (*Syrphidae*, *Diptera*). J Cop Physiol A. 174: 575-579.

Lunau K. 1991. Innate flower recognition in bumblebees (*Bombus terrestris, B. lucorum; Apidae*): optical signals from stamens as landing reaction releasers. Ethology. 88: 203-214.

Lunau K. 1992. Limits of colour learning in a flower-visiting hoverfly, *Eristalis tenax* L. (*Syrphidae, Diptera*). European J Neuroscience, Suppl. 5: 103.

Lunau K. 1995. Notes on the colour of pollen. Pl. Syst. Evol. 198: 235-252.

Lunau K. 2007. Stamens and mimic stamens as components of floral colour patterns. Bot Jahrb Syst. 127: 13-41.

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. Nature Reviews. 13: 758-768.

Morse DH. 1982. The turnover of milkweed pollinia on bumble bees, and implications for outcrossing. Oecologia. 53: 187-196.

Muth M, Papaj DR, Leonard AS. 2015. Bees remember flowers for more than one reason: pollen mediates associative learning. Anim Behav. In-press.

Muth M, Papaj DR, Leonard AS. 2016. Colour learning when foraging for nectar and pollen: bees learn two colours at once. Biol Letters. 11: 20150628. URL http://dx.doi.org/10.1098/rsbl.2015.0628

Naug D, Arathi HS. 2007. Receiver bias for exaggerated signals in honeybees and its implications for the evolution of floral displays. Biol Lett. 3: 635-637.

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 Zool, 46: 197-204.

Pellmyr O. 1985. Pollination Ecology of *Cimicifuga arizonica* (*Ranunculaceae*). Bot Gazette. 146: 404-412.

Raguso 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.

Raine NE, Chittka L. 2007. The adaptive significance of sensory bias in a foraging context: floral colour preferences in the bumblebees *Bombus terrestris.* PLOS one. 6: e556.

Raine NE, Chittka L. 2008. The correlation of learning speed and natural foraging success in bumble-bees. Proc R Soc B. 275: 803-808.

Saleh 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*). PNAS. 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: 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, Kolle M, Andrew P, Chittka L, Steiner U, Glover BJ. 2009a. Floral iridescence, produced by diffractive optics, acts as a cue for animal pollinators. Science. 323: 130-133.

Whitney HM, Chittka L, Bruce TJA, Glover BJ. 2009b. Conical epidermal cells allow bees to grip flowers and increase foraging efficiency. Curr Biol. 19: 948-953.

Wilms J, Eltz T. 2007. Foraging scent marks of bumblebees: footprint cues rather than pheromone signals. Naturwissenschaften. 95: 149–153.

FIGURE LEGENDS

Figure 1. Species of at least 17 families share a morphology termed the solanoid flower form. Members of this type are nectarless, conceal their pollen within functionally poricidal anthers, and often have (human) blue or purple corollas and closely appressed (human) yellow anthers, as in (A) *Commelinaceae*: *Dichorisandra thyrsiflora* (B) *Dilleniaceaea*: *Hibbertia cistiflora* (C) *Elaeocarpaceae*: *Tetratheca* sp. "Flinders" (D) *Gentianaceae*: *Exacum affine* (E) *Gesneriaceae*: *Ramonda myconi* (F) *Iridaceae*: *Ixia scillaris* (G) *Liliaceaea*: *Dianella caerula* (H) *Malvaceae*: *Keraudrenia velutina* (I) *Mayacaceae*: *Mayaca fluviatilis* (J) *Melastomataceae*: *Rhexia petiolate* (K) *Myrsinaceae*: *Ardisia opegrapha* (L) *Pittosporaceae*: *Cheiranthera telfordii* (M) *Pontederiaceae*: *Monochoria vaginalis* (N) *Primulaceae*: *Dodecatheon pulchellum* (O) *Rapateaceae*: *Cephalostemon riedelia* (P) *Solanaceae*: *Solanum melongena* (Q) *Tecophilaceae*: *Conanthera bifolia*. Photographs: A, N: Stan Shebs; B: John Tann; C: Tindo2; D, P: Avery Russell; E: Ferran Turmo Gort; F: Andrew massyn; G: HankyHelper; H: Kevin Thiele2; I, J: Eleanor; K: Reinaldo Aguilar; L: Trex21; M: Jeevan Jose; O: Gustavo Shimizu; Q: Claudio Alvarado Solari. A-N, P-Q licensed by CC BY-NC-SA 2.0; permission for O from author.

Figure 2. (a) Custom-built water tubes used to hold flowers in place during trials. The tube that holds the flower is 3 cm in length. (b) Mosaic and sham flowers constructed from clippings of *S. tridynamum* and *E. affine* flowers. From top left to bottom right: *S. tridynamum* sham, *E. affine* anthers x *S tridynamum* corolla mosaic, *E. affine* sham, and

S. tridynamum anthers x *E affine* corolla mosaic. (c) Removal of the majority of the perianth leaving a circle of corolla tissue joined to stamen filaments for *S. tridynamum* and *E. affine*.

Figure 3. Top panels: Species preference for initially naïve 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 naïve 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 naïve 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 naïve bees. *N*=10 for each experienced treatment. Bottom panel: (e) Species preference for naïve 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 naïve treatment. Letters above bars within a panel indicate significant differences at $p<0.05$ according to a Tukey's post hoc test.

Figure 4. (a) Anther or (b) corolla preference for each species, for naïve and experienced bees visiting arrays consisting of an equal number of *S. tridynamum* and *E. 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.

FIGURES

Figure 1

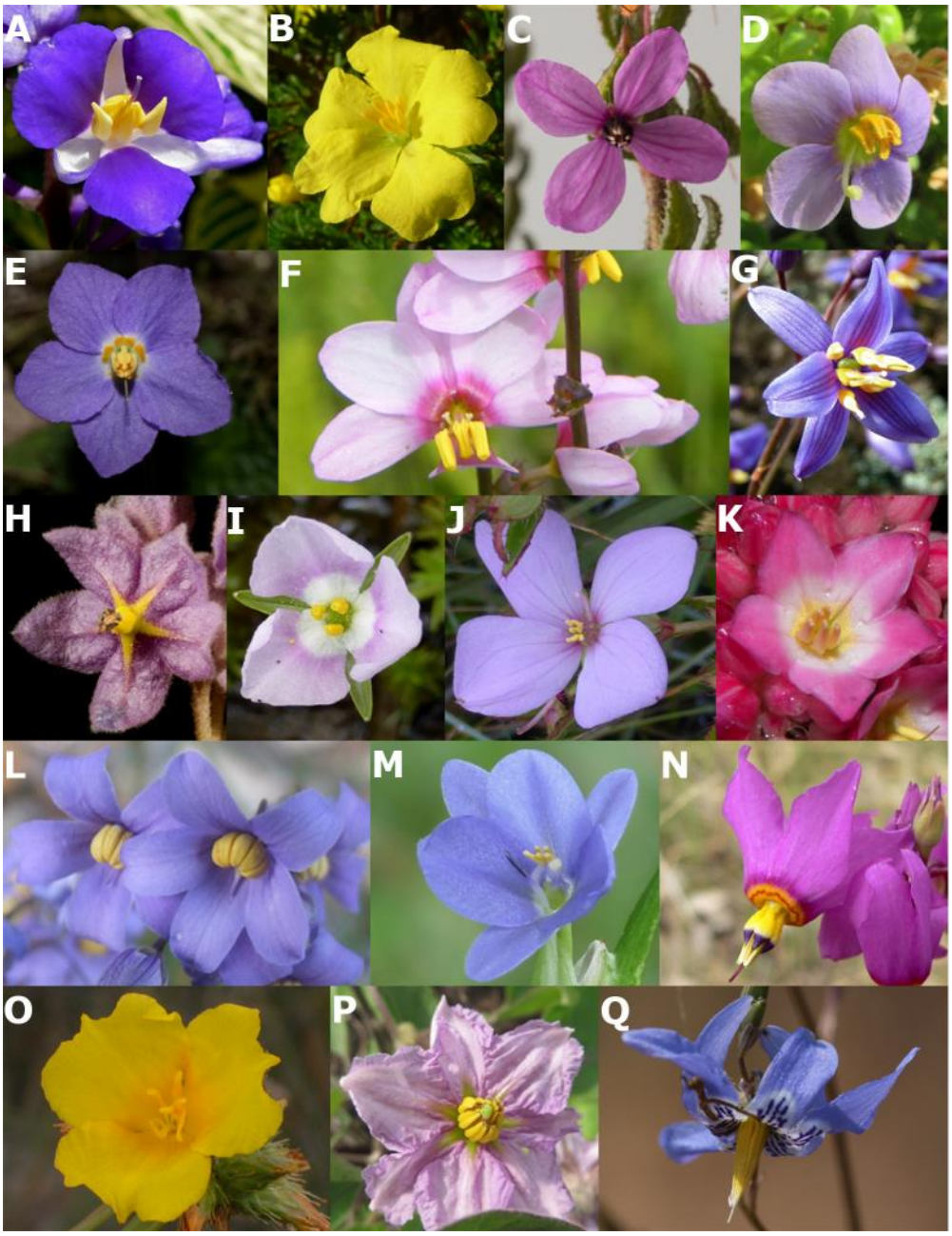


Figure 2a

Figure 2b

Figure 2c

Figure 3

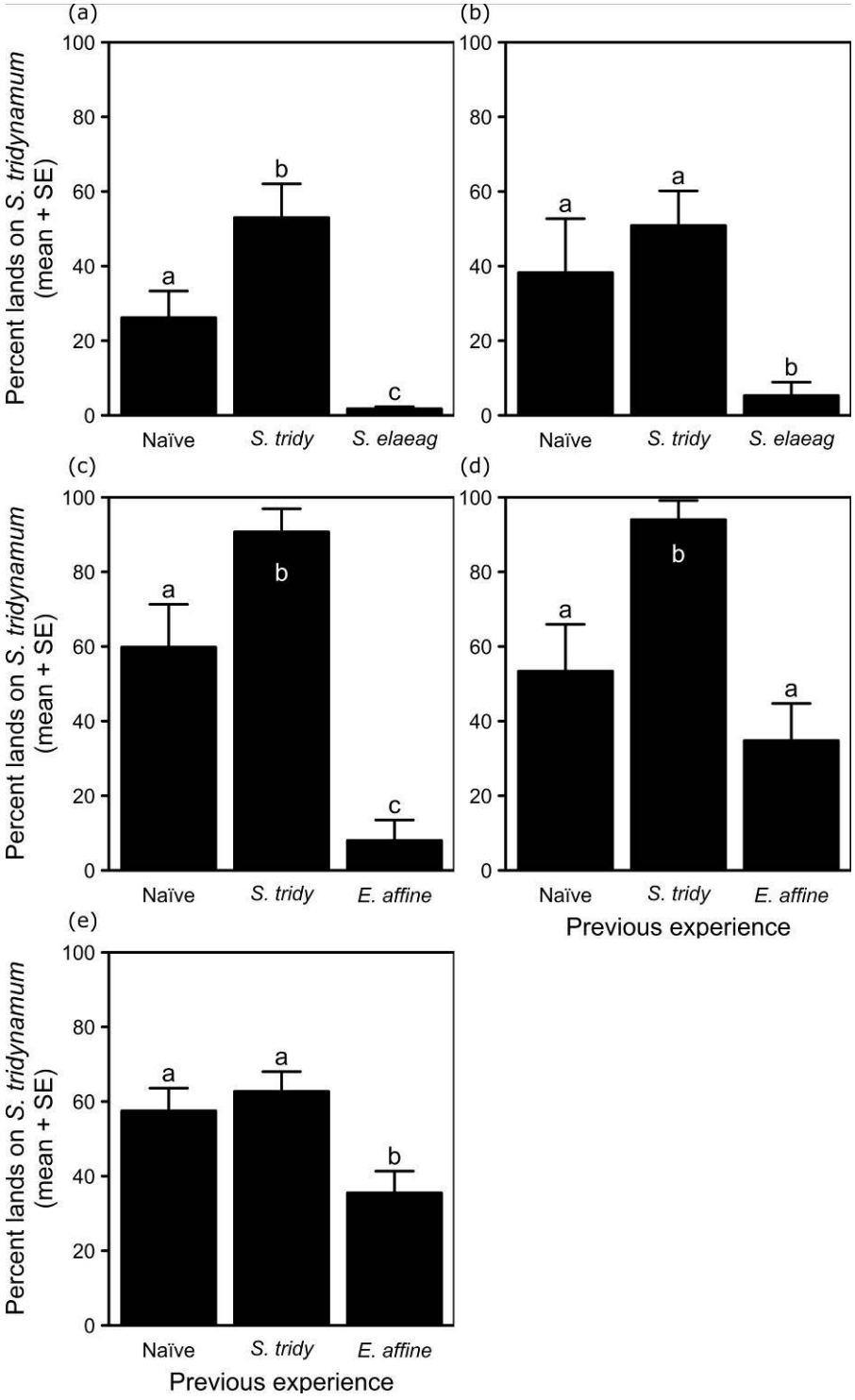
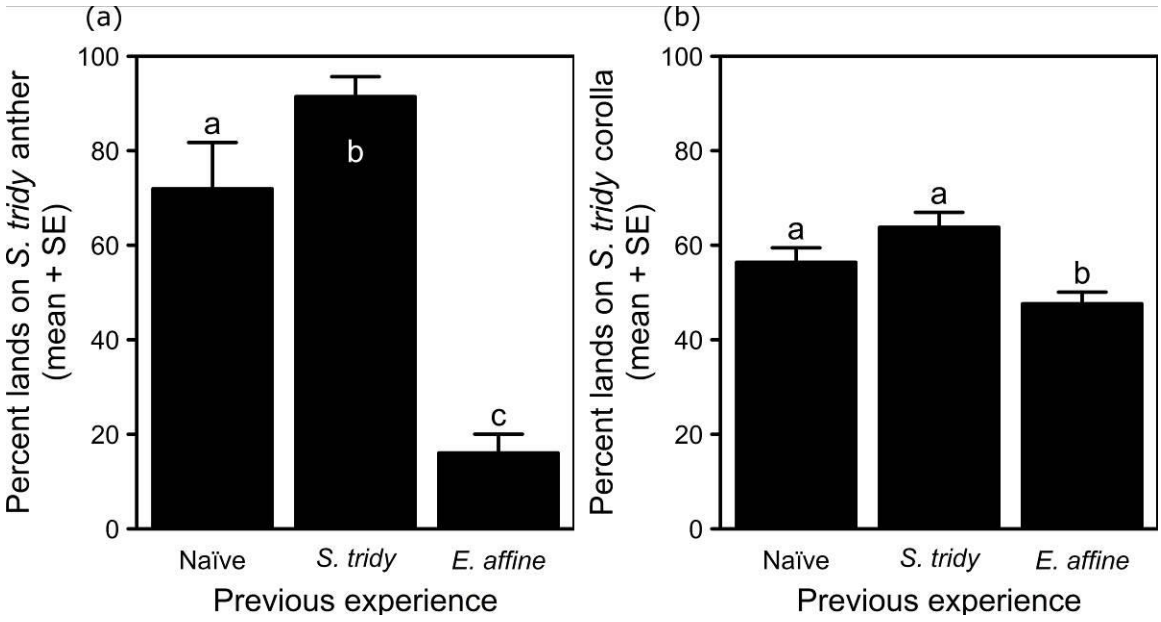

(a)
(b)
(c)
(d)
(e)
Percent lands on S. tridynamum (mean + SE)
100
80
60
40
20
0
a
b
c
Naïve
S. tridy
S. elaeag
E. affine
Previous experience

Figure 4


(a)
Percent lands on S. tridy anther (mean + SE)
100
80
60
40
20
0
a
b
c
Naïve
S. tridy
E. affine
Previous experience
(b)
Percent lands on S. tridy corolla (mean + SE)
100
80
60
40
20
0
a
a
b
Naïve
S. tridy
E. affine
Previous experience

SUPPLEMENTARY MATERIALS

Control for floral tissue splicing

To assess whether cutting flowers and gluing different tissues together affected behavior we examined preference for sham versus intact *S. tridynamum* targets, all of which were made to be unrewarding, as described in the Methods. For the first four trials we verified with a dissecting microscope that anther pores were closed. Bees were presented with a 3 x 3 array lacking a flower in the center position (8 total targets: 4 sham, 4 intact). We found no significant difference in the number of total events or landings (total events, paired *t*-test, $t_7 = 0.260$, $P < 0.802$; landings, paired *t*-test, $t_7 = -0.0201$, $P < 0.984$).

Effects of imbibing sugar water during training

In the event that imbibing sugar water during the short or long term memory training phase affected subsequent pollen-foraging behavior in the testing phase, we removed the 24 bees (out of 104 trained with sugar water in the arena) that had imbibed sugar water to examine whether this had an effect on species preference × experience. We found no changes in significance when compared to tests without these bees removed (GLMERs: Type II Wald χ^2 tests for experience × species choice: short term memory *S. tridynamum* versus *S. elaeagnifolium* $\chi^2 = 22.814$, df = 2, $P < 0.0001$; short term memory *S. tridynamum* versus *E. affine* $\chi^2 = 25.078$, df = 2, $P < 0.0001$; long term memory *S.*

tridynamum versus *S. elaeagnifolium* $\chi^2 = 8.6037$, df = 2, $P < 0.02$; long term memory *S. tridynamum* versus *E. affine* $\chi^2 = 19.715$, df = 2, $P < 0.0001$; short term memory *S. tridynamum* anther versus *E. affine* anther $\chi^2 = 37.737$, df = 2, $P < 0.0001$; short term memory *S. tridynamum* corolla versus *E. affine* corolla $\chi^2 = 9.4323$, df = 2, $P < 0.009$). Likewise, we found no changes in significance in pairwise comparisons from Tukey's post hoc tests.

Effect of scent marking on learned preference

To get an indication of how a bee's scent mark on flowers (c.f. Saleh & Chittka 2006; Wilms & Eltz 2007) might affect its preference for the training species, we filtered the test phase data and the initial species preference data for Experiment 1 to include only the first 10 visits to unvisited (and therefore, unmarked) flowers. We found the same patterns of species preference and no changes in statistical significance for training treatment when compared to tests without this data filtered (Fig. S1; GLMERs: Type II Wald $\chi 2$ tests for experience × species choice: short term memory *S. tridynamum* versus *S. elaeagnifolium* $\chi 2 = 28.175$, df = 2, $P < 0.0001$; short term memory *S. tridynamum* versus *E. affine* $\chi 2 = 42.059$, df = 2, $P < 0.0001$). Likewise, we found no changes in significance in pairwise comparisons from Tukey's post hoc tests (Fig. S1). This analysis demonstrates that the learned species preference is expressed independent of the occurrence of odor marks on flowers.

The first and earliest experience predicts overall preference for naïve bees

To analyze the effect of first choice (either rejection or acceptance) on the non-parametric preference data, we ran Wilcoxon-signed rank tests with 'first rejection choice' or 'first acceptance choice' (of each of the species) as the independent variable and 'preference' (the proportion of landings to one species) as the dependent variable.

For initially-naive bees from Experiment 1, pooled across the short-term and long-term retention treatments, the first rejection choice as well as the first acceptance choice often predicted preference within an array (*S. tridynamum* / *S. elaeagnifolium* arrays: Fig. 2a: Wilcoxon rank sum tests: First rejection choice: $W = 111$, $P < 0.05$; Fig. 2b: First acceptance choice: $W = 129$, $P < 0.003$; *S. tridynamum* / *E. affine* arrays: Fig. 2c: first rejection choice: $W = 58$, $P < 0.119$; Fig. 2d: First acceptance choice: $W = 66$, $P < 0.006$). Thus, even an unrewarding experience can positively influence subsequent preference for that species in a setting where both options are equally available.

Effect of the first and earliest experience on preference for experienced bees

When we examined the short-term preferences of experienced bees (Experiment 1), the first rejection choice and first acceptance choice often predicted preference within an array (*S. tridynamum* / *S. elaeagnifolium* arrays: Fig. S3a: Wilcoxon rank sum tests: First rejection choice: $W = 77$, $P < 0.02$; Fig. S3b: First acceptance choice: $W = 92$, $P < 0.002$;

S. tridynamum / *E. affine* arrays: Fig. S3c: First rejection choice: W = 82, P < 0.0008; Fig. S3d: First acceptance choice: W = 90, P < 0.0004).

When we assayed the long-term preferences of experienced bees (Experiment 1), the first rejection choice and first acceptance choice often predicted preference within an array (*S. tridynamum* / *S. elaeagnifolium* arrays: Fig. S4a: Wilcoxon rank sum tests: First rejection choice: W = 84, P = 0.065; Fig. S4b: First acceptance choice: W = 130.5, P < 0.0001; *S. tridynamum* / *E. affine* arrays: Fig. S4c: First rejection choice: W = 89, P < 0.003; Fig. S4d: First acceptance choice: W = 97, P < 0.0003).

FIGURE LEGENDS

Figure S1. Species preference for initially naïve and experienced bees visiting arrays consisting of an equal number of either (a) *S. tridynamum* and *S. elaeagnifolium* or (b) *S. tridynamum* and *E. affine* in the short term retention tests, when the test phase data was filtered to include only the first 10 visits to unvisited flowers. *N*=14 and 12 for initially naïve bees in the *S. tridynamum* and *S. elaeagnifolium* treatment and *tridynamum* and *E. affine* treatment, respectively. *N*=10 for each experienced treatment. Letters above bars within a panel indicate significant differences at $p<0.05$ according to a Tukey's post hoc test.

Figure S2. Species preference for naïve bees visiting arrays consisting of an equal number of either (a, b) *S. tridynamum* and *S. elaeagnifolium* or (c, d) *S. tridynamum* and *E. affine* and mapped to first rejection (a, c) or acceptance (b, d) choice. *N*=25 and 18 for the *S. tridynamum* / *S. elaeagnifolium* array and *S. tridynamum* / *E. affine* array, respectively. Letters above bars within a panel indicate significant differences at $p<0.05$ according to a Wilcoxon rank sum test.

Figure S3. Species preference for experienced bees visiting arrays consisting of an equal number of either (a, b) *S. tridynamum* and *S. elaeagnifolium* or (c, d) *S. tridynamum* and *E. affine* and mapped to first rejection (a, c) or acceptance (b, d) choice. *N*=25 and 18 for the *S. tridynamum* / *S. elaeagnifolium* array and *S. tridynamum* / *E. affine* array,

respectively. Letters above bars within a panel indicate significant differences at $p<0.05$ according to a Wilcoxon rank sum test.

Figure S4. Species preference for experienced bees visiting arrays consisting of an equal number of either (a, b) *S. tridynamum* and *S. elaeagnifolium* or (c, d) *S. tridynamum* and *E. affine* and mapped to first rejection (a, c) or acceptance (b, d) choice. *N*=25 and 18 for the *S. tridynamum* / *S. elaeagnifolium* array and *S. tridynamum* / *E. affine* array, respectively. Letters above bars within a panel indicate significant differences at $p<0.05$ according to a Wilcoxon rank sum test.

Figure S5. The reflectance spectra of *S. tridynamum* corolla (A1) and anthers (A2), *S. elaeagnifolium* corolla (B1) and anthers (B2), and *E. affine* corolla (C1) and anthers (C2). Spectra of the *S. tridynamum* anthers were created from the yellower proximal area and not the purple distal area of the anthers. Spectra of the corollas of all species were made from the peripheral tissue and not the yellowish central tissue. Representative spectra for all samples was measured using an UV-VIS spectrophotometer (Ocean Optics USB2000) with tungsten-deuterium light source (Ocean Optics DH2000).

Figure S6. The loci in *Bombus impatiens* color space of *S. tridynamum* corolla (A1) and anthers (A2), *S. elaeagnifolium* corolla (B1) and anthers (B2), and *E. affine* corolla (C1) and anthers (C2). Spectra of the *S. tridynamum* anthers were created from the yellower proximal area and not the purple distal area of the anthers. Spectra of the corollas of all species were made from the peripheral tissue and not the yellowish central tissue. The

diagram was made in accordance with Chittka (1992), using data on receptor spectral sensitivities for B. impatiens from Skorupski & Chittka (2010). We used the arena wall on which the flowers were displayed as the background stimulus and the irradiance of the overhead arena lights in calculations of receptor excitation values. Representative spectra for all samples was measured using an UV-VIS spectrophotometer (Ocean Optics USB2000) with tungsten-deuterium light source (Ocean Optics DH2000).

FIGURES

Figure S1

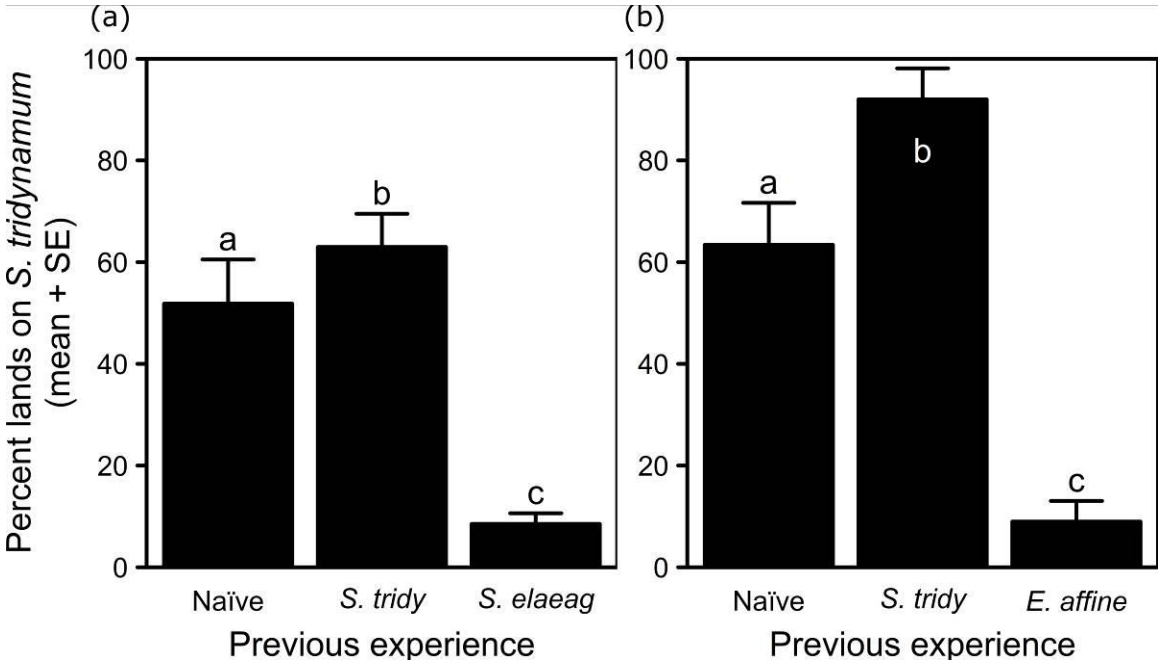


Figure S2

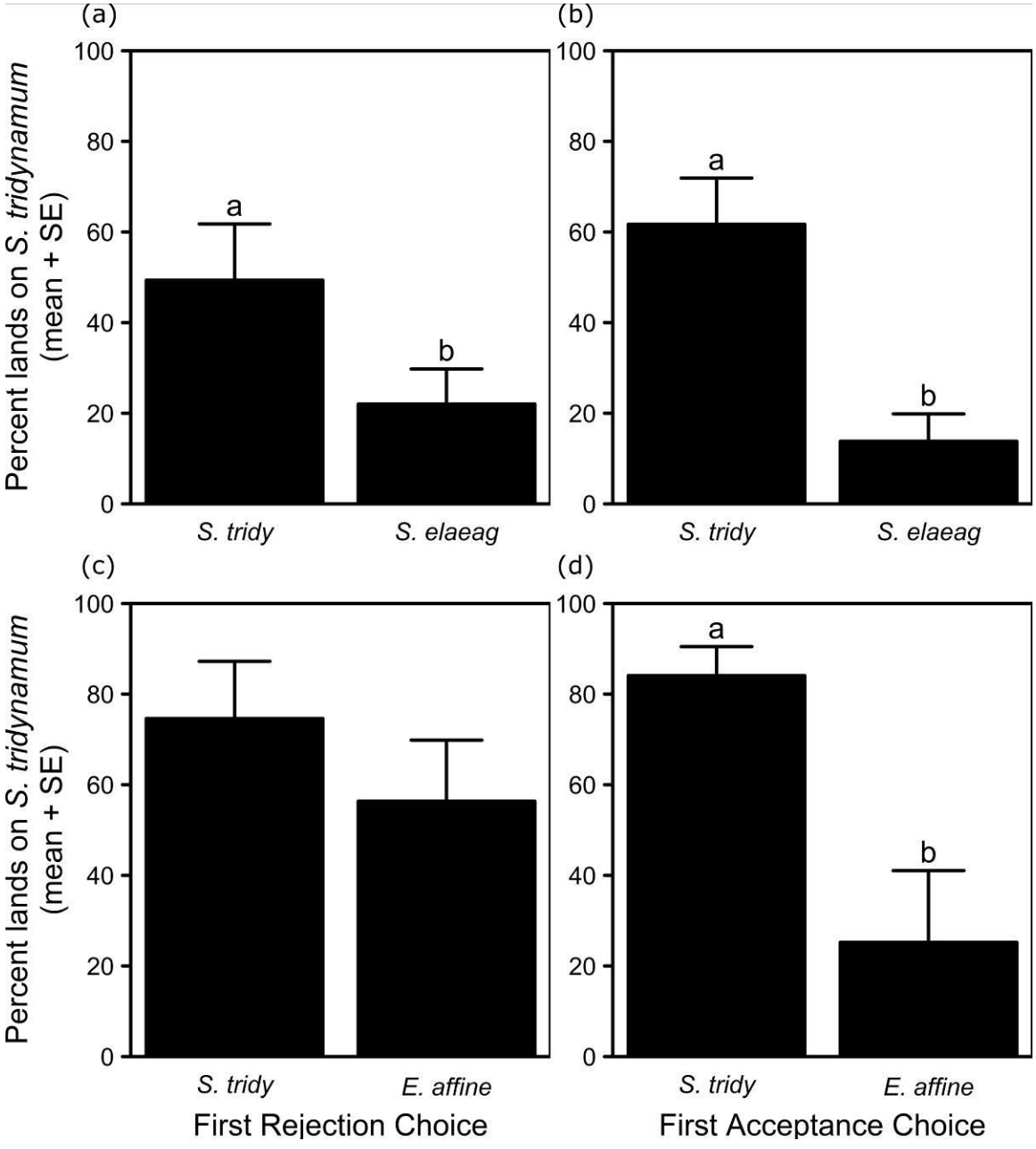

(a)
(b)
(c)
(d)
Percent lands on S. tridynamum (mean + SE)
100
80
60
40
20
0
a
b
S. tridy
S. elaeag
E. affine
First Rejection Choice
First Acceptance Choice

Figure S3

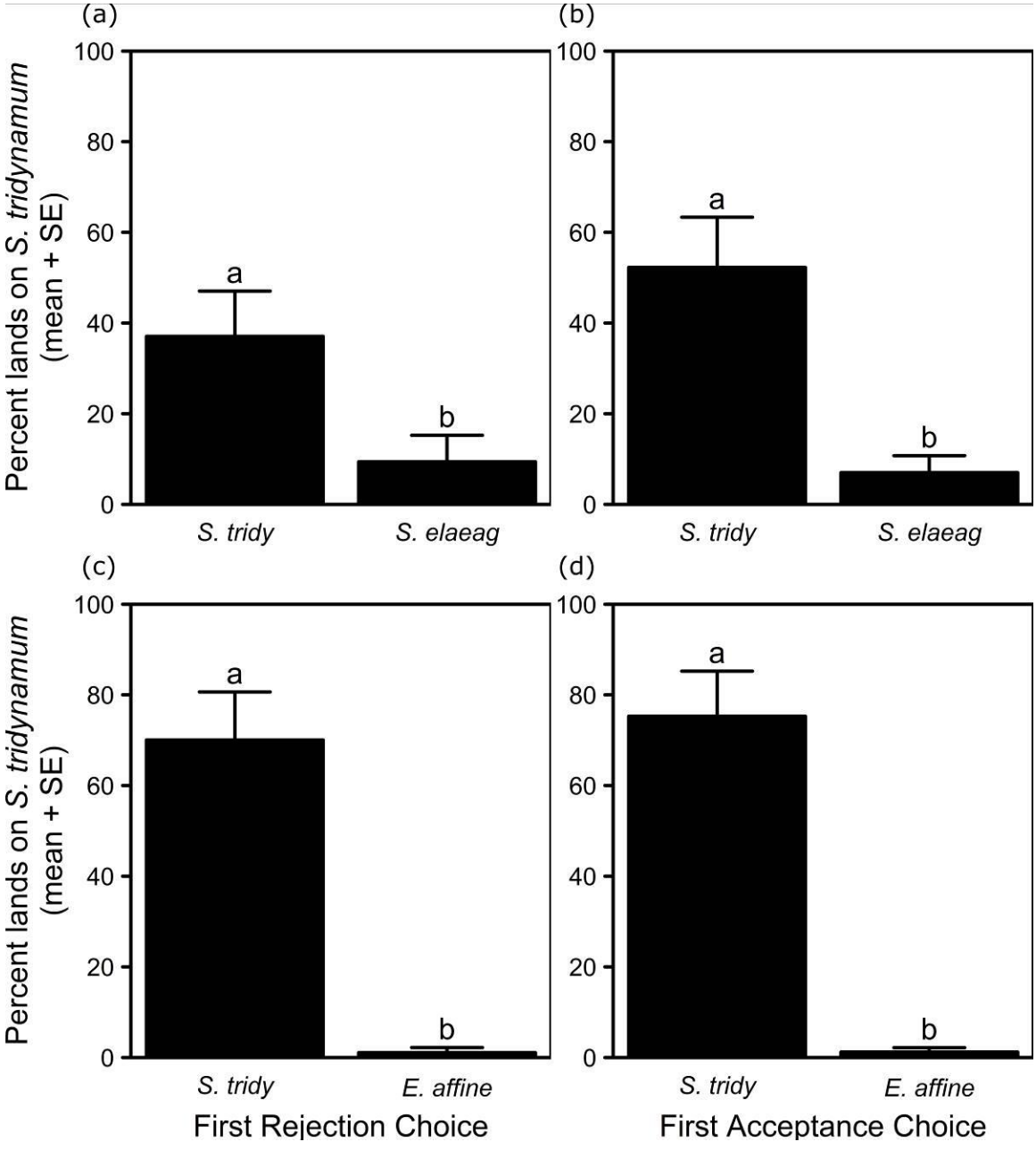

(a)
(b)
(c)
(d)
Percent lands on S. tridynamum (mean + SE)
100
80
60
40
20
0
a
b
S. tridy
S. elaeag
E. affine
First Rejection Choice
First Acceptance Choice

Figure S4

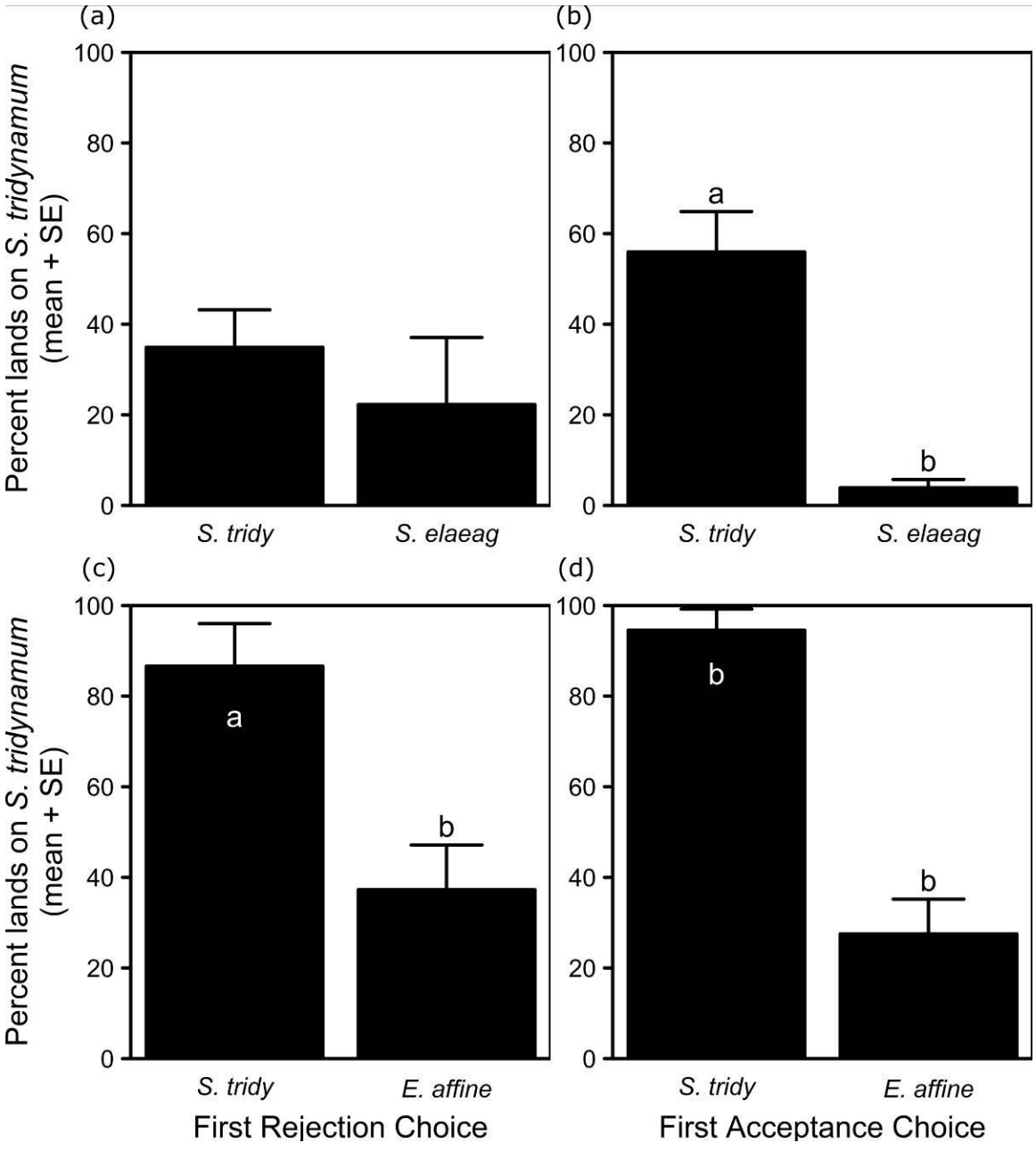

(a)
(b)
(c)
(d)
Percent lands on S. tridynamum (mean + SE)
100
80
60
40
20
0
a
b
S. tridy
S. elaeag
E. affine
First Rejection Choice
First Acceptance Choice

Figure S5

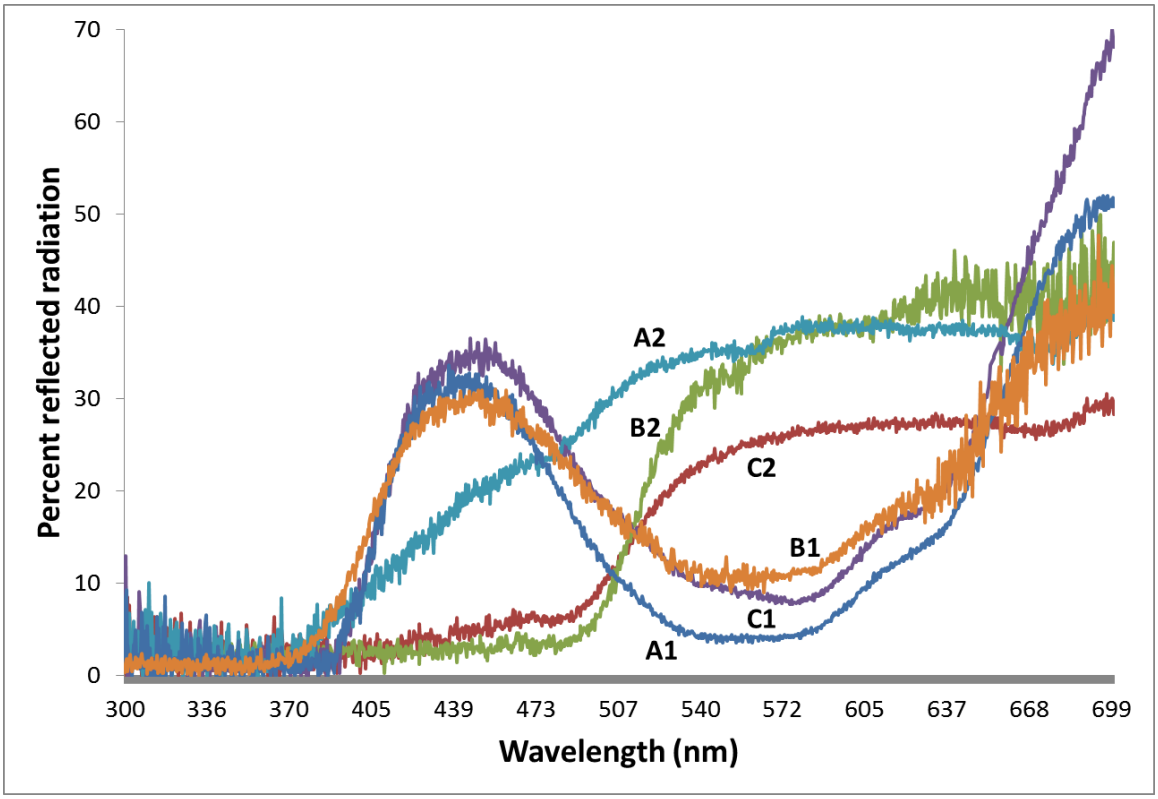
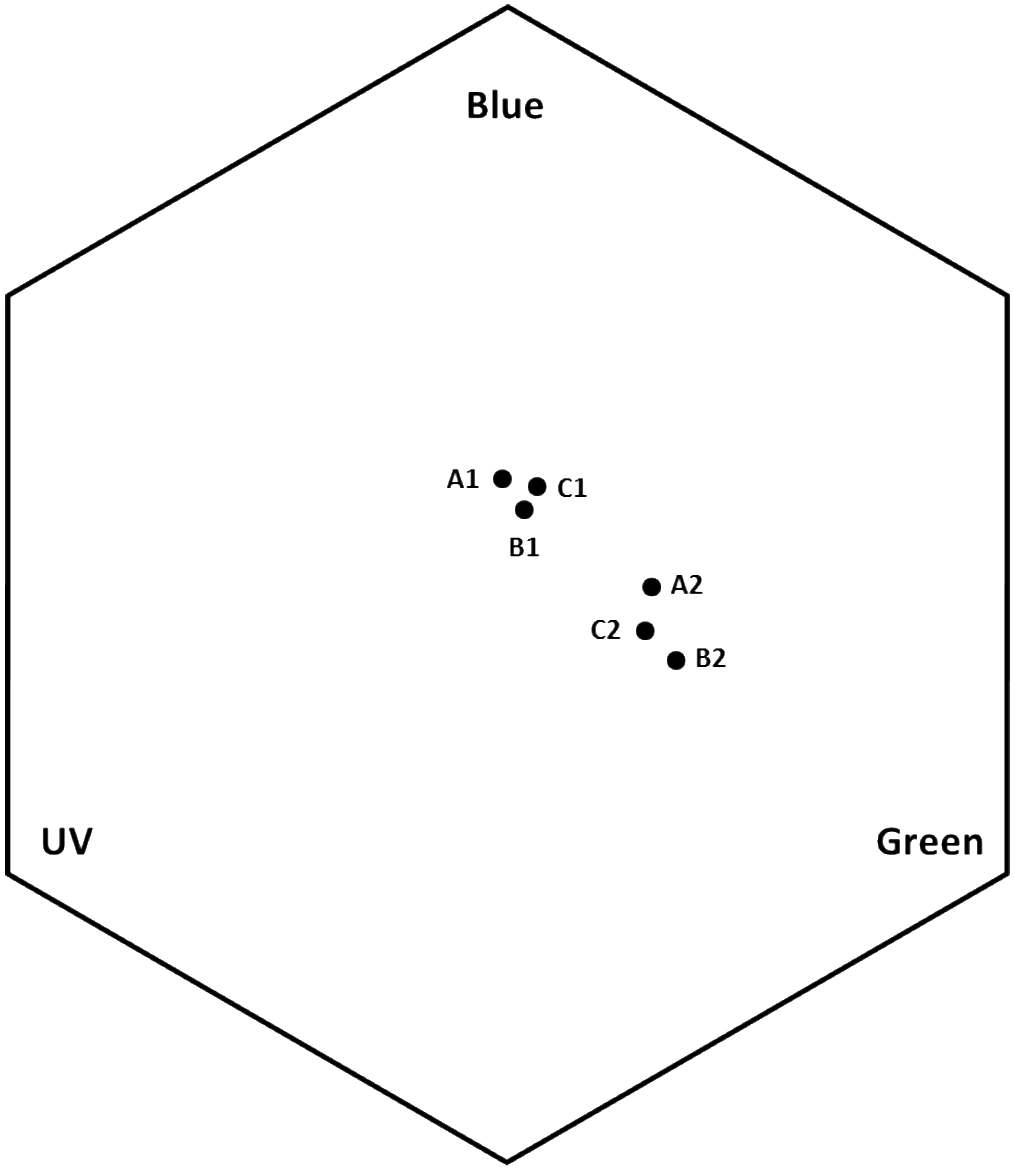

70
60
50
40
30
20
10
0
Percent reflected radiation
300
336
370
405
439
473
507
540
572
605
637
668
699
Wavelength (nm)
A2
B2
C2
B1
C1
A1

Figure S6



APPENDIX C

SYNERGY BETWEEN VISUAL AND OLFACTORY CUES IN ASSESSMENT OF CONCEALED POLLEN REWARDS BY BUMBLE BEES

TITLE: Synergy between visual and olfactory cues in assessment of concealed pollen rewards by bumble bees

Avery L. Russell[ab*], Kevin B. Mauerman[b], Rebekah E. Golden[b], and Daniel R. Papaj[b]

[a] Graduate Interdisciplinary Program in Entomology and Insect Science, University of Arizona, Tucson, AZ, 85721. USA

[b] Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, 85721. USA

*Corresponding author

This manuscript has been formatted for submission to *Animal Behaviour*

ABSTRACT

Flowers frequently advertise their presence, but conceal their rewards. Pollinators often learn floral cues that direct them to concealed nectar. Such learning enables pollinators to forage more efficiently for nectar, but also benefits the plant by encouraging floral fidelity and thus conspecific pollen transfer. Yet, nectar is not the only floral reward. In fact, the pollen of hundreds of thousands of plant species is collected by pollinators such as bees, including the 6-8% of flowering plant species (>22,000 species across >80 families) that conceal pollen within tube-like poricidal floral morphology (e.g., poricidal anthers). Few studies have examined how pollen-offering flowers advertise their presence and, further, direct pollinators to the pollen concealed within the anthers. We examined the signalling function of two floral features, the corolla and the poricidal anthers. We asked if flowers offer cues to the presence of concealed pollen and whether bees learned those cues. We found that visual features of the corolla advertised the flower's presence, while anther chemistry directed nearby bees to find the anthers and to extract the concealed pollen. Pollen chemistry, which could have functioned as a direct cue of concealed pollen, did not elicit pollen extraction behaviour. Instead, chemical cues specifically associated with the anthers provided indirect cues of concealed pollen. Experience mainly modified bees' responses to the anthers and anther chemistry. We conclude that while both anther and corolla have important signalling functions, anther cues are critical to mediating the behaviour of bees foraging for concealed pollen. We discuss the consequences of indirect cues associated with concealed pollen for floral trait evolution, and discuss how these differences in corolla and anther signalling functions

might help explain widespread convergent patterns of floral morphology in plants that offer concealed pollen rewards.

KEY WORDS

Key words: pollen, chemical ecology, concealed rewards, experience, floral signals, indirect cues

HIGHLIGHTS

- Pollen-concealing flowers advertise their presence via corolla visual cues
- Bees use anther indirect cues of concealed pollen to find and extract that pollen.
- Experience mainly modifies bees' responses to the anthers
- Anther cues are not a signal of pollen availability
- The division of signalling functions may explain patterns of floral trait evolution

INTRODUCTION

A central question in animal behaviour is how foraging animals find and exploit resources. Efficient foraging requires that animals locate a resource and ascertain its quality, via cues that can vary in quality and modality (e.g., Prokopy et al. 1986; Raguso 2009). This process of host selection is highly relevant to the study of how pollinators

forage on flowers. Flowering plants signal to pollinators via floral displays that present complex combinations of visual, olfactory, and even electrical cues (Schiestl & Johnson 2013; Muth et al., 2015; Russell et al. 2016a), but floral rewards and direct cues to their quality and presence (e.g., cues intrinsic to the reward such as its scent or visual appearance) are frequently concealed (Lewis 1993; Benitez-Vieyra et al. 2010; Pélabon et al. 2012). Concealment benefits the plant by requiring visitors to search flowers and thereby pollinate flowers, even when rewards may be depleted or absent entirely (Bell 1986). Yet if pollinators could avoid unrewarding flowers before landing on them, they might be able to improve their foraging efficiency and reduce exposure to predators and parasites associated with visiting flowers (Howell & Alarcón 2007).

Floral cues that attract pollinators and indicate the presence of concealed rewards have been studied frequently in the context of pollinators collecting nectar. Plant species that offer nectar rewards often 'advertise' to pollinators with conspicuous floral colours, patterns, and/or scents (Fig. 1A-C; e.g., Raguso 2004; Ye et al. 2011); they also direct nearby pollinators to hidden nectar via markings on the corolla (nectar guides; Fig. 1A-C; e.g., Leonard & Papaj 2011; Hansen et al. 2012; Goodale et al. 2014). Yet, nectar is not the only floral reward: hundreds of thousands of plant species offer pollen rewards to pollinators such as bees, beetles, flies, and some butterflies (Simpson & Neff 1981; Kevan & Baker 1983). Bees in particular must collect pollen, their primary source of protein. Further, c. 6-8% of flowering plant species (>22,000 species, spread across >80 families) conceal their pollen in tube-like poricidal anthers or, less commonly, corollas (Fig. 1D-F; Buchmann 1983; De Luca & Vallejo-Marín 2013; Russell et al. 2016b).

Nevertheless, how bees use floral cues to exploit concealed pollen rewards and implications of this use for floral trait evolution have scarcely been examined.

One possibility is that naïve individuals can use cues of reward presence to find pollen concealed within flowers. This hypothesis requires that pollen-concealing plants offer such floral cues in the first place. Bees might find concealed pollen via direct or indirect floral cues. Because concealed pollen is visually and possibly even olfactorily concealed (Buchmann & Cane 1989; Burkart et al. 2013), direct cues produced by the pollen may be scant. Pollen-concealing flowers may instead provide cues not associated with the pollen (i.e., indirect cues) that direct pollinators to the anthers and, once there, elicit pollen collection behaviour. Even if naïve individuals cannot use such cues, bees may learn to associate floral cues with the presence of the concealed reward. Both possibilities have been shown in a nectar foraging context (e.g., Lunau et al. 2009; Leonard & Papaj 2011; Goodale et al. 2014), but have yet to be demonstrated for bees foraging for concealed pollen. We might expect cues associated with the presence of concealed pollen to be learned, because plants would benefit if bees had to learn such cues. Learning of floral cues is associative, involving enhanced responses to cues paired with the floral reward (Giurfa 2007). Learning is thought to benefit the plant by causing the bee to at least temporarily specialize on that plant species and thereby transfer more conspecific pollen (Waser 1986; Chittka et al. 1999).

An examination of the signalling function of different floral parts should reveal how plants that conceal pollen rewards advertise their presence while also providing direct or

indirect cues of reward presence. Such an examination would in turn shed light on how floral traits have evolved and converged in the thousands of plant species that conceal pollen rewards, as well as how selection on floral traits by pollen-collecting insects differs from that by nectar-collecting insects. In this study, we examined visual and chemical cues used by generalist bumble bees to find and select flowers with poricidal anthers and to locate their concealed pollen. We gave particular attention to two components of the flower, the corolla and the anthers, that might provide such cues. Finally, we examined the role of experience in shaping responses to the various floral features.

METHODS

Bees

We used 141 workers from seven commercially obtained (Koppert Biological Systems, Howell, MI, USA) colonies of the bumble bee *Bombus impatiens* Cresson in laboratory experiments conducted between May 2014 and August 2015. We used approximately equal numbers of bees from each colony and each treatment. We allowed bees to forage daily for sucrose and pollen in arenas constructed of plywood (LxWxH, 82 x 60 x 60cm and 82 x 60 x 30cm). The arenas had clear acrylic ceilings and were lit from above by 40W and 60Hz fluorescent lights (Lithonia Lighting) set to a 14 h :10 h light:dark cycle. Colonies had access to *ad libitum* 2M sucrose solution and pulverized honey bee-collected pollen (Koppert Biological Systems, MI, USA) within the foraging arena.

Sucrose solution was dispensed via braided cotton wicks that extended into vials. Pollen was dispensed via custom-made feeders (Russell & Papaj 2016).

Plants and Flowers

We used freshly clipped flowers from eight *Solanum houstonii* Martyn (synonym: *S. tridynamum*) plants raised under natural light in a university greenhouse with halogen lights used to extend day length to a 14h:10h cycle. Plants were fertilized weekly (Miracle Gro, NPK = 15-30-15). This species offers only pollen rewards (flowers are nectarless) via poricidal anthers. Bees extract the pollen by vibrating the anthers, a process termed floral sonication (Russell et al. 2016b). Flowers from different plants were approximately equally represented in a given trial. We used c. 3778 flowers in experiments.

General Experimental Protocol

All experiments took place in an arena (LxWxH, 82cm x 60cm x 60cm) painted grey on floor and sides. In experiments, freshly clipped flowers were displayed horizontally (their natural orientation) on custom-built water tubes (see Russell et al. 2016c) to prevent desiccation. The water tubes were Velcro-mounted on the arena wall, facing the flight chamber's nest entrance. Flowers were arranged on the wall in a 3 x 3 Cartesian grid with each water tube spaced 7 cm apart in the horizontal and vertical axes of the grid. All test flowers were made unrewarding by gluing the tip of each poricidal anther shut (Elmer's Glue All, Elmer's Products, Inc.). 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, due to an oversight) in time to control for effects of day and time of day on behaviour.

To initiate a trial, we allowed 1-4 flower-naïve workers into the arena simultaneously. When one bee landed on a flower, we immediately removed the others from the arena 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. Bees were always individually trained and tested.

We recorded three behaviours made by bees visiting flowers: approaches, landings without sonication buzzes, and landings with sonication buzzes. An approach was defined as the bee hovering within 3 cm of the flower (often bees would touch the corolla or anthers with their antennae during these approaches): bees always approached flowers before landing. A landing was defined as the bee touching the flower with at least three of its legs simultaneously. Sonication buzzes (or buzzing), which indicated an attempt at extracting pollen, were identified by their distinctive sound and occurred only after a bee had landed (see Russell et al. 2016b for extended description). Additionally, we recorded to which floral tissue (anther, corolla, or surrogate anther) sonication buzzes were delivered, as defined by the placement of the clamped mandibles. We counted the flowers

on which bees landed in each array across all assays: each bee visited most flowers in its array at least once. Sometimes a bee visited the same target more than once in a row. To be conservative, we discarded these repeat visits (across all treatments, an average of 8% of visits) for all analyses, reasoning that such visits may not be independent of the first visits. Bees were allowed to make up to 60 approaches in an array, after which the trial was terminated. A trial was occasionally terminated before the maximum number of approaches if the bee did not forage for a period of five minutes. Most bees made the maximum number of allowed approaches and all bees were included in analyses. Bees were tested only once, after which the bees were euthanized to avoid any transfer of floral cues and *Solanum* pollen to the colony. Flowers were discarded.

To facilitate recording of behaviour, video for all trials was captured at 30fps 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 Lavaliere Microphone, RadioShack) attached to the centre of floral arrays. A Zoom H2 Handy Recorder (ZOOM Corporation) was used to amplify and verify sonication buzzes in ongoing trials.

Experiment 1: Signalling function of corolla and anther

Here we sought to determine how the parts of the whole flower, corolla only and anthers only, mediated foraging behaviour of initially-naïve and experienced bees. This

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

Initially flower-naïve bees were allocated to one of two treatments. In one treatment we assayed initially-naïve behaviour: each bee in this treatment was presented with an array of nine unrewarding flowers. Three types of flowers were represented in each array (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). Flowers were assigned to positions, 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. We did not observe any wilting or browning of the 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 Russell et al. 2016c).

In another treatment we assayed the short-term effects of experience on flower part 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. A divider was then removed and the bee allowed access to the same type of unrewarding nine-flower array described above.

Experiment 2: Importance of corolla visual and olfactory cues

Here we investigated the contribution of corolla visual and olfactory cues to the pollen foraging behaviour of initially-naïve and experienced bees. 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.

We used a protocol modeled after work on hawkmoths by Raguso and Willis (2002, 2005) and on flies by Policha et al. (2016). We allocated initially flower-naïve bees to either of two treatments. In one treatment we assayed initially-naïve behaviour: each bee in this treatment was presented with an array of nine unrewarding flowers. Three types of flowers were represented in each array (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, Virginia, 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, Indiana, U.S.A.; see Raguso & Willis 2005). A clear circular piece of acrylic was added to the backs of all experimental flowers for structural support. To complete the assembly of Bag and Cloth flowers, we hot-glued anthers to the outside of the bag or cheesecloth into where the centre of the flower would be. Flowers were assigned to positions, 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 another treatment, we assayed the short-term effects of experience on flower part 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. A divider was then removed and the bee allowed access to the same type of unrewarding nine flower array described above.

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, oven bag, or no supplementary background (Fig. S2). We confirmed that these backgrounds did not affect bee behaviour (Supplementary Materials, Fig. S3). Furthermore, we examined the spectra of flowers and of the grey 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 foraging arena; likewise, differences in *B. impatiens* perceptual colour space were minimal (Supplementary Materials; Fig. S1).

Experiment 3: Importance of anther versus pollen chemistry

In this experiment tested whether anther and pollen chemical cues mediated foraging behaviour by initially-naïve and experienced bees. To this end, we used surrogate flowers made with real corollas and surrogate foam anthers. Use of the live flower's corolla allowed us to assess whether the anther and pollen extracts affected foraging behaviour. 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.

We made surrogate flowers by cutting off and discarding the stamens from the corollas of flowers. Pure pentane or a pentane anther extract made with *S. houstonii* was applied to Yellow Fibrecraft Foam (Jo-Ann Stores, LLC.), cut into cuboids (LxWxH 1.4 x 0.2 x 0.2cm). These surrogate anthers were hot-glued to the corollas, resulting in a mosaic Surrogate Anther flower (Fig. 2G). Surrogate Anther flowers were arranged in a 3 x 3 grid without a central flower (eight total targets), with pentane control and extract-treated targets alternated by position. See Supplementary Material for details on extract and surrogate anther flower preparation.

Initially flower-naïve bees were allocated to any of three treatments. In two treatments we assayed initially-naïve behaviour by presenting bees with surrogate flowers arranged in a 3 x 3 grid without a central flower (eight total targets), with pentane control and extract-treated targets alternated by position. Extracts in one treatment were made from *S. houstonii* pollen and from *S. houstonii* anthers in the other treatment. In the third treatment we assayed the short-term effects of experience collecting pollen from *S. houstonii* by first training and then immediately testing a separate group of bees. Training followed the same protocol described for previous experiments. After training, bees were allowed access to the same type of unrewarding eight flower array as described above. After completion of a trial, each bee in the third treatment was labelled with individually numbered plastic coloured tags (The Bee Works) 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 (Supplementary Materials, Fig. S4).

Data Analyses

All data were analysed using R v.3.2.0 (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 et al. 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-α (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 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 amongst three or more categories it is the only statistical approach to our knowledge that does not suffer from inflated type I/II error rates when analysing differences between categories that (a) are not independent, (b) lack moderate correlation between dependent variables, (c) have outliers, (d) and do not have homogeneity of variances.

Variables analysed in experiment 3 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 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 rank tests. We also analysed whether naïve bees on their first landing choice had preferences for one flower type over another, using Chi-square (χ^2) 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

Initially-naïve bumble bees (*Bombus impatiens*) tended to approach *Solanum houstonii* flowers that had an intact corolla (Fig. 3A). There was a highly significant statistical difference in approaches between flowers with corollas (Sham or Corolla flowers) and flowers without (Anther flowers) (Fig. 3A, Table 1; Bayesian analysis: Sham versus Anther, $PP < 0.0001$; Corolla versus Anther, $PP < 0.0001$). Additionally, there was a trend for initially-naïve bees to approach Sham flowers more frequently than Corolla flowers (Fig. 3A, Table 1; Bayesian analysis: Sham versus Corolla, $PP = 0.053$).

Bumble bees that had prior experience collecting pollen from *S. houstonii* likewise tended to approach *S. houstonii* flowers that had an intact corolla, with experience enhancing bees' approach responses to the anthers (Fig. 3A). Experienced bees

approached flowers with corollas more frequently than flowers without corollas (Fig. 3A, Table 1; Bayesian analysis: Sham versus Anther, $PP < 0.0001$; Corolla versus Anther, $PP < 0.0001$). Experienced bees also approached Sham flowers more frequently than Corolla flowers to a small but significant extent (Fig. 3A, Table 1; Bayesian analysis: Sham versus Corolla, $PP = 0.041$). Experienced bees made a much greater proportion of approaches to these two types over flowers that had only anthers (Anther flowers) (Fig. 3A, Table 1; Bayesian analysis: Sham versus Anther, $PP < 0.0001$; Corolla versus Anther, $PP < 0.0001$). Comparing the responses of initially-naïve to experienced bumble bees, experienced bees more frequently approached Anther flowers by a small but significant degree (Fig. 3A, Table 1; Bayesian analysis, initially-naïve versus experienced: Sham, $PP = 0.223$; Corolla, $PP = 0.248$; Anther, $PP < 0.007$).

Results for landings were similar in some respects to results for approaches, but with important differences. As with approaches, initially-naïve bees made a greater proportion of their landings on Sham flowers than on Corolla flowers; however, in this case, the difference was strongly significant (Fig. 3B, Table 1; Bayesian analysis: Sham versus Corolla, $PP < 0.0001$). As with approaches, initially-naïve bees made proportionately more landings on flowers with corollas (Sham or Corolla flowers) than on flowers that had anthers, but lacked a corolla (Anther flowers), differences that were again highly significant (Fig. 3B, Table 1; Bayesian analysis: Sham versus Anther, $PP < 0.0001$; Corolla versus Anther, $PP < 0.0001$).

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 Sham flowers relative to flowers that had either their corolla or anthers removed, and landed much more frequently on flowers that lacked a corolla over flowers that lacked anthers (Fig. 3B, Table 1; Bayesian analysis: Sham versus Corolla, $PP < 0.0001$; Sham versus Anther, $PP < 0.0001$; Corolla versus Anther, $PP < 0.0001$). Furthermore, relative to initially-naïve bees, experienced bees landed much more frequently on flowers that had anthers (Sham and Anther flowers) and a much smaller proportion of landings on Corolla flowers (Fig. 3B, Table 1; Bayesian analysis, initially-naïve versus experienced: Sham, $PP < 0.0003$; Corolla, $PP < 0.0001$; Anther, $PP < 0.0001$).

Lastly, the corolla barely elicited pollen extraction behaviour, regardless of experience level: for both experienced and initially flower-naïve bees (pooled) visiting flowers, we observed that 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 corolla visual cues

Both initially naïve and experienced bumble bees (*B. impatiens*) mainly approached *S. houstonii* flowers with intact corolla visual and olfactory cues (Sham flowers) over flowers that lacked one of these features (Fig. 4A). Further, naïve and experienced bees

both made significantly fewer approaches to flowers that lacked corolla visual cues (Cloth flowers) than to the two flower types that had intact corolla visual cues (Sham or Bag flowers) (Fig. 4A, Table 2; Bayesian analysis: initially-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, $PP < 0.0001$).

The pattern of landing responses was similar to the pattern of approaches, with one exception. As with approaches, bees of both experience levels tended to make a greater proportion of landings on Sham flowers over flowers lacking visual cues (Cloth flowers) or odour cues (Bag flowers); however, the difference between Sham flowers and Bag flowers was significant only for initially-naïve bees (Fig. 4B, Table 2; Bayesian analysis: initially-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, $PP < 0.0001$).

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 corolla visual cues (Bag flowers) significantly less frequently, relative to initially-naïve bees, although this difference was numerically small (Fig. 4B, Table 2; Bayesian analysis, initially-naïve versus experienced: Sham, $PP = 0.225$; Corolla, $PP = 0.345$; Anther, $PP < 0.042$).

Experiment 3: Bees use cues associated with the anthers to find and extract concealed pollen

Anther chemistry did not affect approaches: both initially-naïve bees and experienced bees were equally attracted to anther extract-treated surrogate flowers and pentane-treated surrogate flowers (Fig. 5A; 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, $t_{15.39} = -0.263$, $P < 0.796$).

Conversely, initially-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 control (Fig. 5A; paired *t*-test: naïve bees, extract versus pentane, $t_{10} = 3.900$, $P < 0.003$; Bonferroni correction α-value = 0.025). Surrogate flowers consisted of live *Solanum* corollas bearing artificial foam anthers treated with the pentane extract or control. Furthermore, after experience collecting pollen from *S. houstonii*, this difference became significantly more pronounced (Fig. 5A; Welch two sample *t*-test: naïve versus experienced, $t_{23.02} = -2.736$, $P < 0.012$; Bonferroni correction α-value = 0.025). These patterns were also mirrored by the first landing choice of bees: for their first choice, more naïve and experienced bees landed on anther extract-treated surrogate flowers than pentane control treated surrogate flowers, although this difference was only significant for experienced bees (χ^2-test for first landing choice: naïve bees, $\chi^2_1 = 2.273$, $P = 0.132$, $N = 11$ bees: 72.7; experienced bees, $\chi^2_1 = 5.4$, $P < 0.021$, $N = 15$ bees; percent that made their first landing on anther extract; naïve bees: 72.7%; experienced bees: 80.0%).

Furthermore, chemical extracts made from *S. houstonii*'s anthers, but not of its pollen, elicited pollen extraction behaviour by initially-naïve bumble bees (Fig. 5B). A significantly greater proportion of initially-naïve bees' approaches ended in sonication on flowers treated with anther extract versus those treated with pollen extract (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 initially-naïve bees visiting surrogate flowers treated with a pollen extract versus surrogate flowers treated with a pentane control (Fig. 5B; paired *t*-test, pollen extract versus pentane: $t_{14} = 1.94$, $P < 0.073$). On visits where bees buzzed anther-extract treated flowers, sonication 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 (Fig. 5C; Wilcoxon signed rank test: anther extract versus pentane, % lands with sonication to the surrogate anther, $W = 80$, $P < 0.0006$).

Results summary

Our findings demonstrate that the corolla and anthers of *S. houstonii* serve different signalling functions for bumble bees foraging for concealed pollen. In terms of approaches and landings, initially flower-naïve foragers relied primarily on the corolla. Experienced bees likewise relied primarily on the corolla to first locate flowers. Pollen extraction behaviour (floral sonication) by initially-naïve and experienced bees was

elicited by contact with the poricidal anthers. However, experience with rewarding *S. houstonii* flowers greatly altered bees' responses in other ways. In contrast to initially-naïve bees, for example, for experienced bees the anthers assumed a key role in mediating landings. Additionally, we found that differences in signalling function between corolla and anthers extend to differences in signalling modality. The responses of initially-naïve bees to the corolla were mediated primarily by visual cues, while bee responses to the anther appear to be mediated by anther chemistry at a minimum. Furthermore, experience with rewarding *S. houstonii* flowers greatly heightened bees' responses to anther chemistry, but only modestly affected bees' responses to the corolla. Finally, anther chemistry appears to serve as an indirect cue of concealed pollen: extracts of *S. houstonii* pollen (a direct cue) did not elicit sonication by initially-naïve bees, but extracts of the flower's anthers (an indirect cue) readily elicited floral sonication behaviour.

DISCUSSION

Cues that indicate the presence of nectar enable pollinators to forage more efficiently, for instance by allowing them to find concealed nectar faster and avoid potentially unrewarding flowers (Howell & Alarcón 2007; Leonard & Papaj 2011; Hansen et al. 2012). Such cues can also benefit the plant, particularly when cues of nectar presence are indirect (e.g., not intrinsic to the reward, such as its scent or visual appearance). Indirect cues encourage pollinators to explore the flower even when nectar may be depleted (Bell

1986). Indirect cues of nectar presence can be learned (Leonard & Papaj 2011). Such learning is thought to encourage short term specialization (floral fidelity), which increases the likelihood of pollen transfer to conspecific plants (Waser 1986; Chittka et al. 1999). Although many pollinator taxa collect both pollen and nectar (Kevan & Baker 1983; Nicolson 2011), indirect cues of reward presence have only been studied in the context of nectar collection. Yet the benefits to plant and pollinator of indirect cues should not depend on whether the reward being offered is pollen or nectar. We therefore expect plants that conceal either type of reward to offer indirect cues as to the presence of the reward. Our results suggest that indirect cues of reward presence are indeed offered by plants with concealed pollen rewards. Furthermore, with experience, bumble bees become adept at using these indirect cues to locate concealed pollen.

Because indirect cues of concealed pollen are used, they could conceivably contribute to floral fidelity, and thereby benefit the plant. However, the benefits of these indirect cues to the bee are less clear. In previous work we showed that with experience, bees made only modest gains in accessing pollen from poricidal anthers (Russell et al. 2016b). Furthermore, the present results suggest that indirect cues were not indicators of pollen presence. Specifically, our results suggest anther chemistry - and not pollen chemistry - elicited attempts by bees to extract the concealed pollen. Thus, anther chemistry might serve to entice bees into landing and wasting considerable time and energy sonicating even completely drained (but still attractive) anthers, potentially enhancing conspecific pollen transfer at the cost of the bee. Yet there could be other as unexplored ways that anther chemistry might serve as an honest cue of pollen presence. For instance, the

attractiveness of anther chemistry to pollen-foraging bees could be developmentally tied to pollen depletion, analogous to how floral colour changes are hypothesized to signal to nectar-foraging pollinators that nectar rewards have been depleted (Weiss 1991, 1995).

The importance of anther cues in mediating pollen foraging behaviour might also help explain why, for instance, sterile anthers and staminodes are common amongst pollen-offering species (Walker-Larsen & Harder 2000; Wang & Hu 2011 and references within). For instance, the functionally female flowers of cryptically dioecious species have anthers, but release no or only sterile pollen (Wang & Hu 2011 and references within). Sterile anthers, rather than being retained as a result of developmental or genomic constraints, have been suggested to function as lures that encourage nearby bees to explore even rewardless flowers and thereby transfer pollen to them (Lunau 2007; Penny 2014). Such a strategy would be even more effective if the plant were able to conceal cues of pollen reward presence from pollinators (Benitez-Vieyra et al. 2010). Cryptically dioecious species frequently hide their pollen within poricidal anthers (e.g., Knapp et al. 1996), and several studies, including the present one, suggest that such pollen may not be detected by bee pollinators prior to its extraction from the anthers (Buchmann & Cane 1989; Burkart et al. 2013). Concealment within poricidal anthers might even facilitate the evolution of pollen deceit generally and cryptic dioecy specifically (Vogel 1978). If pollinators are indeed unable to ascertain whether a poricidal flower contains pollen prior to alighting and exploring the flower, as our study suggests, the evolution of poricidal flowers that invest fewer and fewer resources into pollen production should be favoured.

The responses of pollen foragers also shed light on the evolution of other patterns of floral morphology common amongst nectarless plant species. For instance, bee-pollinated species in at least 17 families of flowering plants exhibit a convergent pattern of floral morphology called the solanoid flower form (Faegri 1986; De Luca & Vallejo Marin 2013; Russell et al. 2016c). Solanoid flowers resemble a bullseye; the typically (human) blue or purple corolla is reflexed, exposing a central grouping of enlarged poricidal and anthers that are typically yellow (to the human eye). Our findings suggest that signalling functions are segregated between these two floral structures. In particular, the corolla of solanoid flowers functions to attract pollen foragers. The anthers on the other hand function to direct nearby bees to locate the anthers and, once there, to elicit pollen extraction behaviour. Might the recurrent evolution of the solanoid form reflect selection for highly apparent anthers by pollen-foraging bees? The reflexed corolla of solanoid flowers provides a largely unobstructed view of the closely packed anthers, as well as a frequently strong visual contrast to the enlarged anthers; contrast might be further enhanced if the anthers differed from the corolla in other sensory modalities. For instance, preliminary data suggests that the anthers of *S. houstonii* are at least olfactorily distinct from the corolla (A. Russell, A. Kessler, S. Buchmann, & D. Papaj unpub. data).

Our results have important general implications for the study of floral signalling. It is well understood that plants signal their pollinators via floral displays that present complex combinations of visual, olfactory, tactile, and even electrical cues (Giger & Srinivasan 1995; Gumbert 2000; Whitney et al. 2009; Whitney et al. 2009; Clarke et al.

2013; Schiestl & Johnson 2013; Foster et al. 2014). These so-called multimodal displays serve a variety of signalling functions; for instance, they enable pollinators to find flowers from a distance, assess reward presence, and determine where to probe for concealed nectar (e.g., Raguso & Willis 2005; Pélabon et al. 2012; Goodale et al. 2014). Yet particular signalling functions sometimes appear to rely primarily on a single sensory modality (e.g., Raguso 2004; Raguso & Willis 2002, 2005). Our results suggest that different floral structures can also serve diverse signalling functions, exploiting different sensory modalities. Specifically, floral advertisement was achieved mainly through corolla visual cues, while anther chemistry served as a close-range cue to bumble bees, eliciting landing and pollen collection. Flowers are complex structures and the qualities of their signals can vary across different floral parts (e.g., Dobson et al. 1996; Burdon et al. 2015). Future work would benefit by integrating an understanding of how multimodal cues and signalling functions are segregated across different floral structures, in order to better understand the function and evolution of floral display traits.

In conclusion, pollinator responses are thought to contribute to the evolution of a variety of floral display traits in a nectar foraging context, including corolla shape, colour, markings, and scent (Van der Niet & Johnson 2012; Schiestl & Johnson 2013). Learned responses are particularly interesting, because they can involve even evolutionary novel stimuli and thus may have contributed significantly to the rapid diversification of floral form (Lewis 1993; Chittka & Thomson 2005; Leonard et al. 2011; Hopkins & Rausher 2012; Russell et al. 2016c). In this study, experience with pollen-only flowers led bumble bees to adopt strong and long-lasting preferences for features of the anthers. Surprisingly,

experience only modestly affected responses to the corolla, suggesting that bees foraging for pollen respond to different floral features than bees foraging for nectar. We suggest that the responses of pollen-foraging bees such as those we detected in this study may have contributed to the diversity of anther form, particularly for plant species in which pollen is the main or sole reward offered to pollinators. Future work should examine how pollen and nectar foraging pollinators might contribute to selection on different floral features for plant species that offer both pollen and nectar rewards. Likewise we should examine the extent to which floral features for these species share signalling functions that both pollen and nectar foraging pollinators use.

FUNDING

This work was supported by the Graduate & Professional Student Council of the University of Arizona and the National Science Foundation (IOS-1257762).

ACKNOWLEDGEMENTS

We are grateful to Anne Leonard for helpful comments, to Abreeza Zegeer for greenhouse care, and to Sarah White and Eleni Moschonas for assistance in running experimental trials.

REFERENCES

Benitez-Vieyra, S., Ordano, M., Fornoni, J., Boege, K., & Domínguez, C. A. (2010). Selection on signal–reward correlation: limits and opportunities to the evolution of deceit in *Turnera ulmifolia* L. S. *Journal of Evolutionary Biology, 23*, 2760-2767.

Buchmann, S. L., & Cane, J. H. (1989). Bees assess pollen returns while sonicating Solanum flowers. *Oecologia, 81*, 289-294.

Buchmann, S. L. (1983). Buzz pollination in angiosperms. In C. E. Jones, & R. J. Little (Eds.), Handbook of experimental pollination biology (pp. 73-113). New York, NY: Van Nostrand Reinhold.

Burdon, R. C. F., Raguso, R. A., Kessler, A., Parachnowitsch, A. L. (2015). Spatiotemporal floral scent variation of *Penstemon digitalis*. *Journal of Chemical Ecology, 41*, 641-650.

Burkart, A., Schlindwein, C., & Lunau, K. (2013). Assessment of pollen reward and pollen availability in *Solanum stramoniifolium* and *Solanum paniculatum* for buzz-pollinating carpenter bees. *Plant Biology, 16(2),* 503-7

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

Chittka, L., Thomson, J. D., & Waser, N. M. (1999). Flower constancy, insect psychology and plant evolution. *Naturwissenchaften, 86*, 361-377.

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

De Luca, P. A., & Vallejo-Marín, M. (2013). What's the "buzz" about? The ecology and evolutionary significance of buzz-pollination. *Current Opinion in Plant Biology, 16*, 429-435.

Dobson, H. E. M., Groth, I., & Bergström, G. (1996). Pollen advertisement: chemical contrasts between whole-flower and pollen odors. *The American Journal of Botany, 83*, 877-885.

Faegri, K. (1986). The solanoid flower. *Transactions and proceedings of the Botanical Society of Edinburgh, 45*, 51-59.

Fordyce, J. A., Gompert, Z., Forister, M. L., & Nice, C.C. (2011). A hierarchical bayesian approach to ecological count data: a flexible tool for ecologists. PLoS ONE 6(11). DOI: 10.1371/journal.pone.0026785

Forister, M. L., & Scholl CF (2012) Use of an exotic host plant affects mate choice in an insect herbivore. *The American Naturalist, 179(6),* 805-810.

Foster, J. J., Sharkey, C. R., Gaworska, V. A., Roberts, N. W., Whitney, H. M., & Partridge, J. C. (2014). Bumblebees learn polarization patterns. *Current Biology, 24,* 1415-1420.

Giger, A. D., & Srinivasan, M. V. (1995). Pattern recognition in honeybees: eidetic imagery and orientation discrimination. *Journal of Comparative Physiology A, 176,* 791-795.

Giurfa, M. (2007). Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. *Journal of Comparative Physiology A, 19*3, 801-824.

Gompert, Z., & Fordyce, J. A. (2012). BayesPref: heirarchical Bayesian analysis of ecological count data. http://CRAN.R-project.org/package=bayespref. (accessed May 2016)

Goodale, E., Kim, E., Nabors, A., Henrichon, S., & Nieh, J.C. (2014). The innate responses of bumble bees to flower patterns: separating the nectar guide from the nectary changes bee movements and search time. *Naturwissenschaften*. DOI: 10.1007/s00114-014-1188-9

Gumbert, A. (2000). Color choices by bumble bees (*Bombus terrestris*): innate preferences and generalization after learning. *Behavioral Ecology and Sociobiology, 48,* 36-43.

Hansen, D. M., Van der Niet, T., & Johnson, S. D. (2012). Floral signposts: testing the significance of visual 'nectar guides' for pollinator behaviour and plant fitness. *Proceedings of the Royal Society B, 279,* 634-639.

Hopkins, R., & Rausher, M. D. (2012). Pollinator-mediated selection on flower color allele drives reinforcement. *Science, 335,* 1090-1092.

Howell, A. D. & Alarcón, R. (2007). Osmia bees (Hymenoptera: Megachilidae) can detect nectar-rewarding flowers using olfactory cues. *Animal Behaviour, 74,* 199-205.

Kevan, P. G. & Baker, H. G. (1983). Insects as flower visitors and pollinators. *Annual Review of Entomology, 28,* 407-453.

Knapp, S., Persson, V., & Blackmore, S. (1998). Pollen morphology and functional dioecy in *Solanum* (Solanaceae). *Plant Systematics and Evolution, 210,* 113-139.

Leonard, A. S., Papaj, D. R. (2011). 'X' marks the spot: the possible benefits of nectar guides to bees and plants. *Functional Ecology, 25,* 1293-1301.

Leonard, A. S., Dornhaus, A., & Papaj, D. R. (2011b). Why are floral signals complex? An outline of functional hypotheses. In S. Patiny, editor. Evolution of plant-pollinator relationships (pp. 279-300). MA: Cambridge University Press.

Lewis, A.C. (1993). Learning and the evolution of resources: pollinators and flower morphology. In D. R. Papaj & A. C. Lewis (Eds.), Insect learning: Ecological and evolutionary perspectives (pp. 219–242). New York, NY: Chapman & Hall.

Lunau, K. (2007). Stamens and mimic stamens as components of floral colour patterns. *Botanische Jahrbücher fur Systematik, 127*, 13-41.

Lunau, K., Unseld, K., & Wolter, F. (2009). Visual detection of diminutive floral guides in the bumblebee *Bombus terrestris* and in the honeybee *Apis mellifera*. *Journal of Comparative Physiology A, 195*, 1121-1130.

Muth, F., Papaj, D. R., & Leonard, A. S. (2015). Colour learning when foraging for nectar and pollen: bees learn two colours at once. *Biology Letters, 11*, 20150628. DOI: http://dx.doi.org/10.1098/rsbl.2015.0628

Nicolson, S. W. (2011). Bee food: the chemistry and nutritional value of nectar, pollen and mixtures of the two. *African Zoology, 46(2),* 197-204. DOI: http://dx.doi.org/10.1080/15627020.2011.11407495

Pélabon, C, Thöne, P, Hansen, T. F., & Armbruster, W. S. (2012). Signal honesty and cost of pollinator rewards in *Dalechampia scandens* (Euphorbiaceae). *Annals of Botany, 109*, 1331-1339.

Penny, R. H. (2014). Sexual dimorphism in cryptically dioecious *Thalictrum macrostylum*. International Journal of Plant Sciences, 175(7), 794-802.

Policha, T., Davis, A., Barnadas, M., Dentinger, B. T. M., Raguso, R. A., and Roy, B. A. (2016). Disentangling visual and olfactory signals in mushroom-mimicking *Dracula* orchids using realistic three-dimensional printed flowers. *Functional Ecology*, DOI: 10.1111/nph.13855

Prokopy, R. J., Papaj, D. R., Cooley, S. S., & Kallet, C. (1986). On the nature of learning in oviposition site acceptance by apple maggot flies. *Animal Behaviour, 34*, 98-107.

R Core Development Team. (2010). R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Raguso, R. A. & Willis, M. A. (2002). Synergy between visual and olfactory cues in nectar feeding by naïve hawkmoths, *Manduca sexta*. *Animal Behaviour, 64,* 685-695.

Raguso, R. A. & Willis, M. A. (2005). Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, *Manduca sexta*. *Animal Behaviour, 69,* 407-418.

Raguso, R. A. (2004). Why are some floral nectars scented. *Ecology, 85(6),* 1486-1494.

Raguso, R. A. (2009). Floral scent in a whole-plant context: moving beyond pollinator attraction. *Functional Ecology, 23,* 837-840.

Russell, A. L. & Papaj, D. R. (2016). Artificial pollen dispensing flowers and feeders for bee behaviour experiments. *Journal of Pollination Ecology, 18,* 13-22.

Russell, A. L., Newman, C. R., & Papaj, D. R. (2016a). White flowers finish last: Pollen-foraging bumble bees show biased learning in a floral color polymorphism. *Evolutionary Ecology,* DOI: 10.1007/s10682-016-9848-1

Russell, A. L., Leonard, A. S., Gillette, H. D., & Papaj, D. R. (2016b). Concealed floral rewards and the role of experience in floral sonication by bees. *Animal Behaviour, 120*, 83-91.

Russell, A. L., Golden, R. E., Leonard, A. S., & Papaj, D. R. (2016c). Bees learn preferences for plant species that offer only pollen as a reward. *Behavioral Ecology, 27,* 731-740.

Schiestl, F. P. & Johnson, S. D. (2013). Pollinator-mediated evolution of floral signals. *Trends in Ecology and Evolution, 28*, 307-315.

Simpson, B. B. & Neff, J. T. (1981). Floral rewards: alternatives to pollen and nectar. *Annals of the Missouri Botanical Garden, 68(2*), 301-322.

Van der Niet, T & Johnson, S. D. (2012). Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology and Evolution, 27(6),* 353-361.

Vogel, S. (1978). Evolutionary shifts from reward to deception in pollen flowers. In Richards AH, editor. The pollination of flowers by insects (pp. 89-96). London: Academic Press.

Walker-Larsen, J. & Harder, L. D. (2000). The evolution of staminodes in angiosperms: patterns of stamen reduction, loss, and functional re-invention. *American Journal of Botany. 87,* 1367-1384.

Wang, Y.-C. & Hu, J.-M. (2011). Cryptic dioecy of *Symplocos wikstroemiifolia* Hayata (Symplocaceae) in Taiwan. *Botanical Studies, 52*, 479-491.

Waser, N. M. (1986). Flower constancy: definition, cause and measurement. *The American Naturalist, 127(5)*, 593-603.

Weiss, M. R. (1991). Floral colour changes as cues for pollinators. *Nature, 354*, 227-229.

Weiss, M. R. (1995). Floral color change: a widespread functional convergence. *The American Journal of Botany, 82(2)*, 167-185.

Whitney, H. M., Kolle, M., Andrew, P., Chittka, L., Steiner, U., & Glover, B. J. (2009a). Floral iridescence, produced by diffractive optics, acts as a cue for animal pollinators. *Science, 323*, 130-133.

Wood, S. (2015). Mixed GAM computation vehicle with GCV/AIC/REML smoothness estimation (R package version 1.9-9). https://stat.ethz.ch/R-manual/R-devel/library/mgcv/html/mgcv-package.html (accessed May 2016)

Ye, X.-Q., Meng, J.-L., Zhao, Z.-G, Fan, B.-L, & Du, G.-Z. (2011). Optimal pollinator attraction strategies in *Trollius ranunculoides* Hemsl. (Ranunculaceae) at different altitudes: increased floral display or promotion of nectar output? *Plant Biology*, DOI: doi:10.1111/j.1438-8677.2010.00402.x

FIGURE LEGENDS

Figure 1. The complex floral displays of various species. Three species with prominent floral displays and which direct nearby pollinators to hidden nectar via conspicuous visual markings on the corolla: (A) *Ipomoea hederacea* (B) *Chilopsis linearis* (C) *Kallstroemia grandiflora*. Three species conceal pollen either within a very prominent poricidal corolla: (D) *Pedicularis groenlandica*; or very prominent poricidal anthers (E) *Dodecatheon meadia* (F) *Solanum dulcamara*. Photographs: (A-C,F): Avery Russell; (E): Yvi Russell by permission; (D) Walter Siegmund, licensed by CC BY-SA 3.0.

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), and (F) corolla concealed under cheesecloth and anthers glued to the outside (Cloth flower). Experiment 3: (G) surrogate foam anther glued to corolla (Surrogate Anther flower). Flowers in D-F backed by acrylic disk.

Figure 3. Percentage of responses (+SE) by initially-naïve and experienced bumble bees (*Bombus impatiens*) to flowers (*Solanum houstonii*) that had a corolla and anthers (Sham flowers), a corolla only (anthers had been removed; Corolla flowers), or anthers only (corolla had been removed; Anther flowers). (A) Approaches and (B) landings. $N = 31$, 21 for initially-naïve and experienced bees, respectively. 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.

Figure 4. Percentage of responses (+SE) by initially-naïve and experienced bumble bees (*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. *N* = 15, 14 for initially-naïve and experienced bees, respectively. 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. Mean percentage of responses (+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 initially-naïve and experienced bees. *N* = 11,15 for initially-naïve and experienced bees, respectively. Dashed line at 50% indicates random expectation for an assay with two choices. (B) Mean percentage of approaches (+SE) to surrogates treated with anther extract or pure pentane that resulted in sonication. *N* = 11,15 initially-naïve bees for anther and pollen extract treatment, respectively. Anther extract bees were from the same treatment as in (A). (C) Mean percentage of landings with sonication that specifically involved buzzes to the surrogate anthers versus the corolla for anther-extract treated surrogate flowers versus pentane treated surrogate flowers. *N* = 8 initially-naïve bees; from the same treatment as in (A).

Asterisks and different letters above bars within a panel indicate significant differences at $p<0.05$ according to *t*-tests or a Wilcoxon signed rank test.

FIGURES & TABLES

Table 1: Experiment 1, differences in posterior probabilities ('*PP*)' via a Bayesian analysis

	Naïve: Approaches	Naïve: Landings	Experience: Approaches	Experience: Landings	Naïve vs Experience: Approaches	Naïve vs Experience: Landings
Sham vs Corolla	*PP* = 0.053	*PP* < 0.0001	*PP* = 0.041	*PP* < 0.0001		
Sham vs Anther	*PP* < 0.0001	*PP* < 0.0001	*PP* < 0.0001	*PP* < 0.0001		
Corolla vs Anther	*PP* < 0.0001	*PP* < 0.0001	*PP* < 0.0001	*PP* < 0.0001		
Sham					*PP* = 0.223	*PP* < 0.0003
Corolla					*PP* = 0.248	*PP* < 0.0001
Anther					*PP* < 0.007	*PP* < 0.0001

Table 1: Experiment 2, differences in posterior probabilities ('*PP*)' via a Bayesian analysis

	Naïve: Approaches	Naïve: Landings	Experience: Approaches	Experience: Landings	Naïve vs Experience: Approaches	Naïve vs Experience: Landings
Sham vs Bag	*PP* = 0.044	*PP* = 0.018	*PP* = 0.038	*PP* = 0.059		
Sham vs Cloth	*PP* < 0.0001	*PP* < 0.0001	*PP* < 0.0001	*PP* < 0.0001		
Bag vs Cloth	*PP* < 0.0001	*PP* < 0.0001	*PP* < 0.0001	*PP* < 0.0001		
Sham					*PP* = 0.225	*PP* = 0.432
Bag					*PP* = 0.345	*PP* = 0.226
Cloth					*PP* < 0.043	*PP* = 0.094

Figure 1

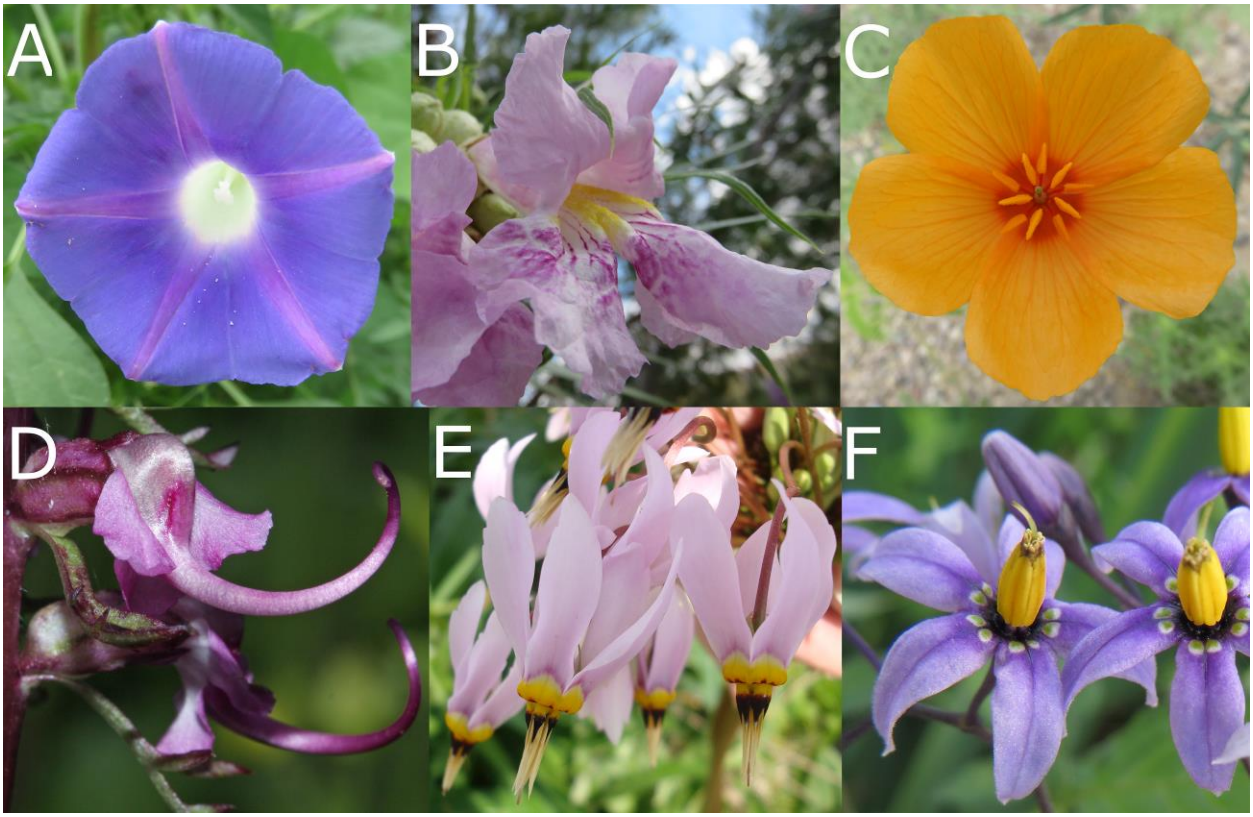


Figure 2

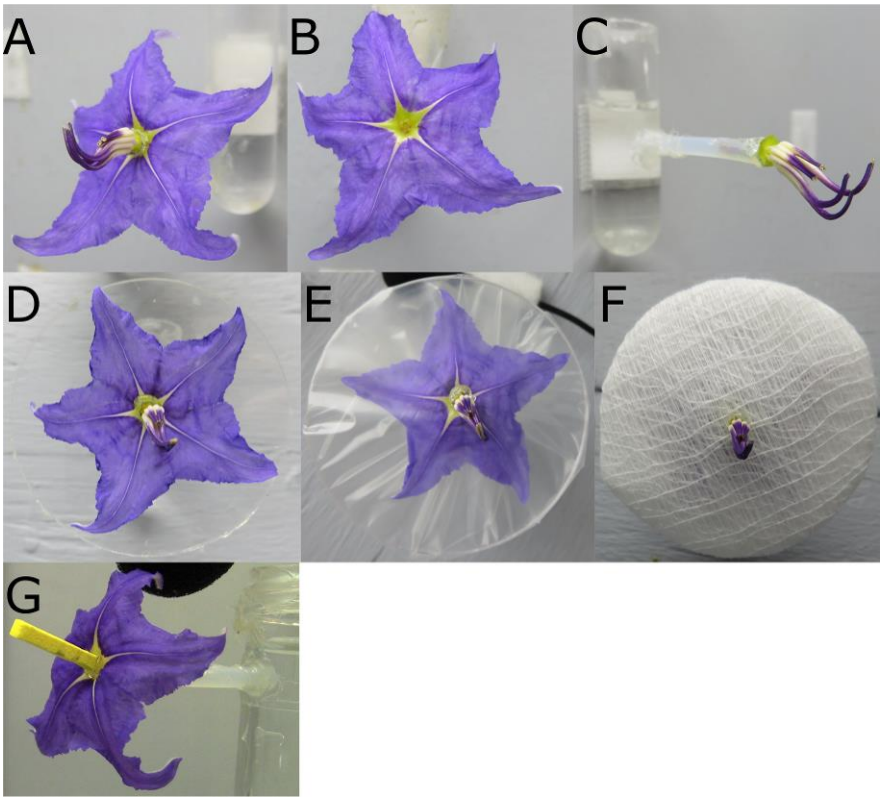


Figure 3

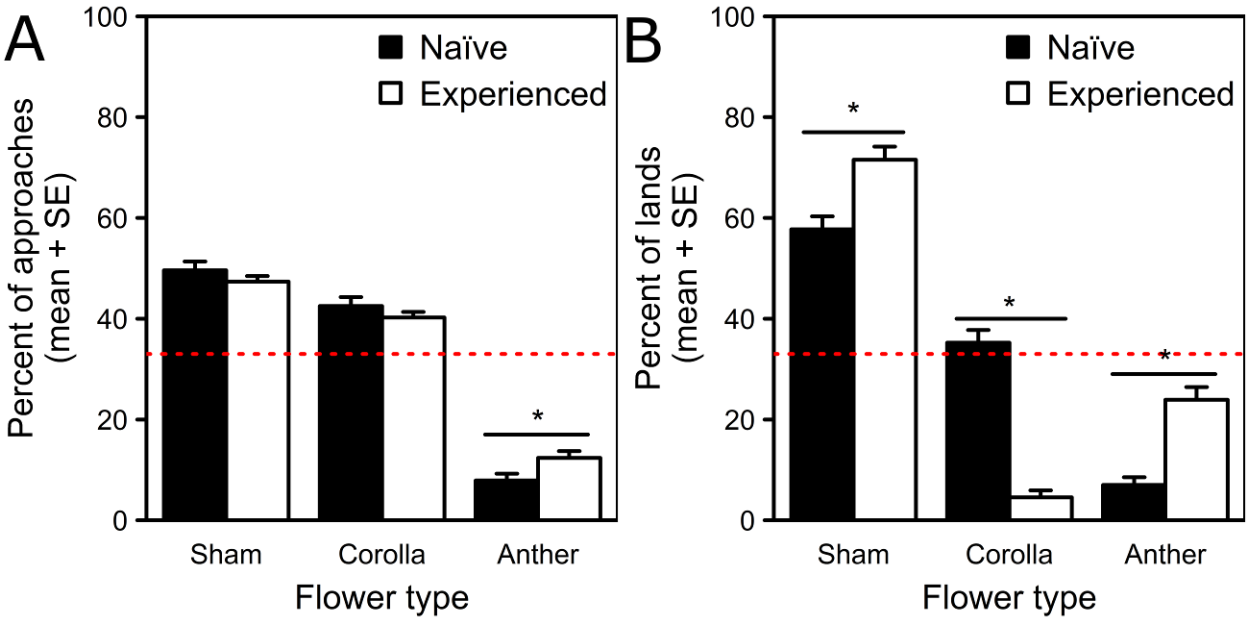


Figure 4

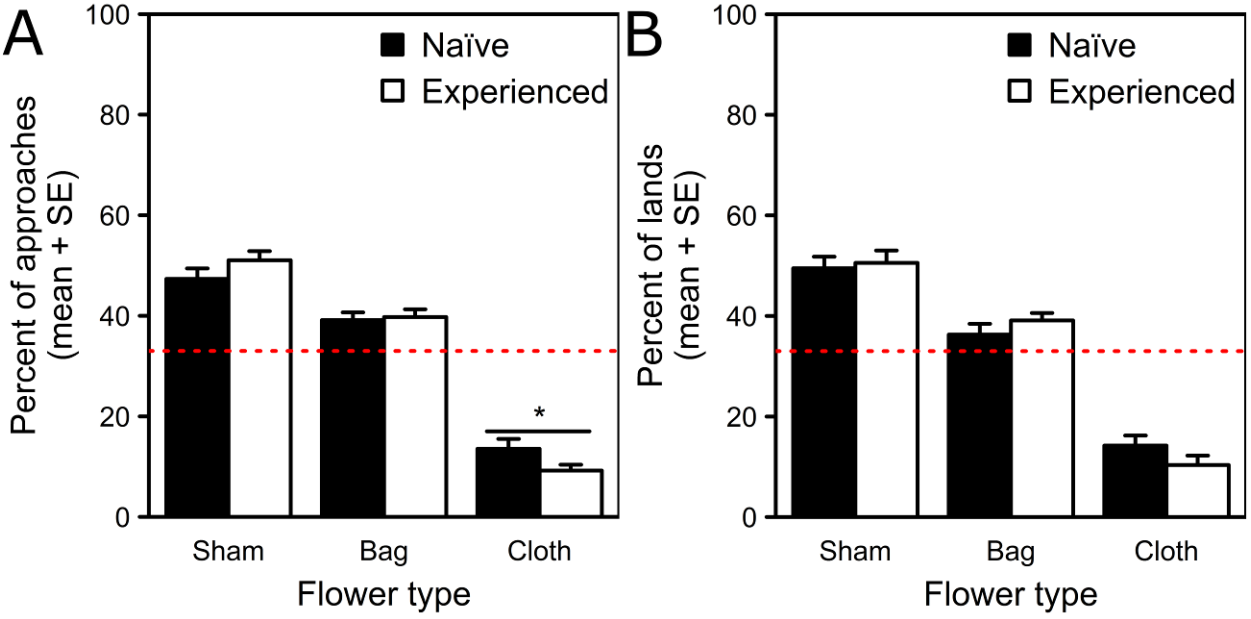
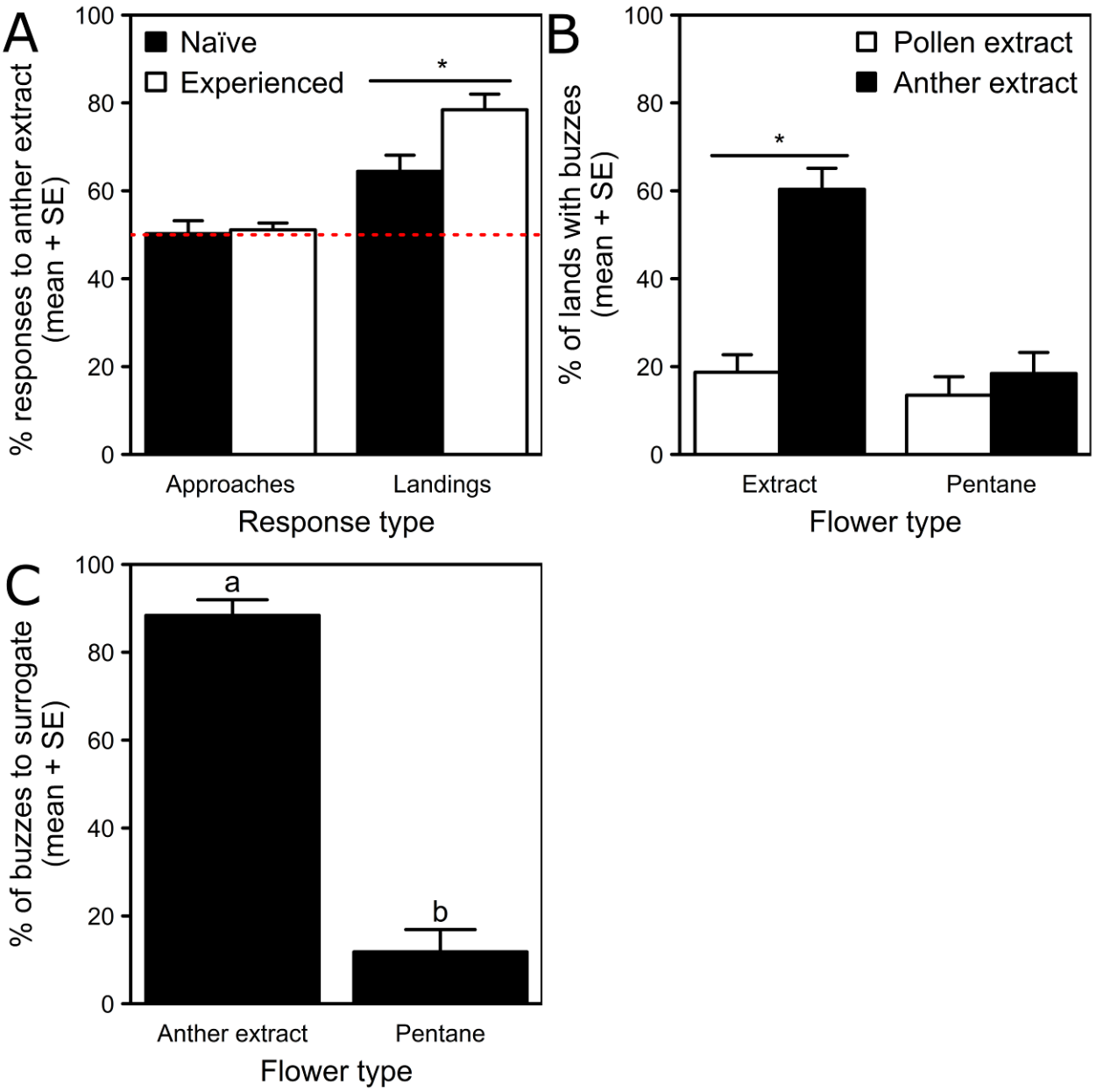


Figure 5


A
Naïve
Experienced
*
% responses to anther extract (mean + SE)
100
80
60
40
20
0
Approaches
Landings
Response type
B
Pollen extract
Anther extract
*
% of lands with buzzes (mean + SE)
Extract
Pentane
Flower type
C
a
b
% of buzzes to surrogate (mean + SE)
Anther extract
Pentane
Flower type

SUPPLEMENTARY MATERIAL

SI METHODS

Preparation of surrogate flowers and pentane anther extracts

1.5 mL of Pentane HPLC Grade (Fisher Scientific Company, LLC.) was added to a sterile 4 dram glass vial (BioQuip Products, Inc CA, USA). Each anther extract was made from 60 flowers of *Solanum houstonii*. The anthers of 20 flowers (100 *S. houstonii* anthers) were excised at the filament base and submerged within the pentane for 5 minutes. Anthers were removed from the vial with sterile forceps. An amount of pentane equal to that which had evaporated when the anthers were removed (typically ~0.2mL) was added to the vial. These steps were repeated until the pentane extract contained an equivalent of 60 flowers' anthers (~600 anthers) in 1.0mL of solvent. 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 (2.01mg pollen/flower when the study was performed). We used pollen frozen at -18 C that had been removed from the flowers of three *S. houstonii* plants on December 4th & 8th, 2013 using an ultrasonicator (Virtis Virsonic 100, Boston Laboratory Equipment). Using a Sartorius Analytic Balance (Data Weighing Systems, Inc.) we weighed out 120 mg of pollen and placed the pollen in a sterile 2.0mL amber autosampler vial (Thermo Fisher Scientific Inc.). We added 1.2 mL of Pentane HPLC Grade (Fisher Scientific Company, LLC.) to the vial. The pollen

extract thus contained an equivalent of 60 flowers' pollen in 1.0mL of solvent. Anther and pollen extracts were transferred to separate sterile 2.0mL amber autosampler vials (Thermo Fisher Scientific Inc.). Prepared extracts were stored at -18 C.

Yellow Fibrecraft Foam (Jo-Ann Stores, LLC.) was cut into cuboids (LxWxH 1.4 x 0.2 x 0.2cm) that were about the length of the *Solanum* stamens. 0.1mL pure pentane or anther extract were applied to each foam surrogate anther via 1mL Pyrex disposable serological pipettes (Sigma-Aldrich Co. LLC.). When the solvent had evaporated (the foam conveniently turns white for ~1 second upon evaporating), the surrogate was ready to be glued in place. The anthers of flowers were excised and an extract-treated surrogate hot-glued at this spot (Fig. 2G). This order is critical, as pentane solvent otherwise destroys floral tissue (A. Russell, pers. obs.). We discarded surrogate flowers after 30 minutes if unvisited, in case the anther extract lost its attractiveness to the bees with time.

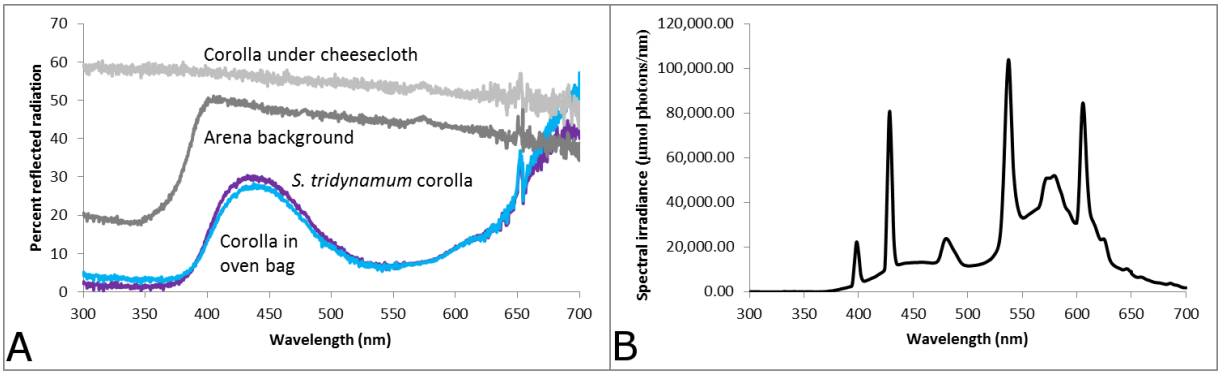
Reflectance and irradiance spectra and bee colour space

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. S1). Each reflectance spectrum consists of the mean of five measurements. Each measurement was taken from a flower (or part of the foraging arena). Reflectance spectra for all samples were measured using an UV–VIS spectrophotometer (Ocean Optics USB2000) with tungsten-deuterium light source (Ocean Optics DH2000) and a

fluoropolymer white standard (USRS-99-010 Spectralon; Labsphere, NH, USA). 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 flight arena was measured at the center of the foraging array using a P600 UV/VIS optical fiber (Ocean Optics), a CC-3-UV-T cosine-corrected (180 degrees) irradiance probe (Ocean Optics), and a tungsten-deuterium calibration light source (Ocean Optics DH2000). 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 & Chittka (2010). We used the 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.

Figure S1



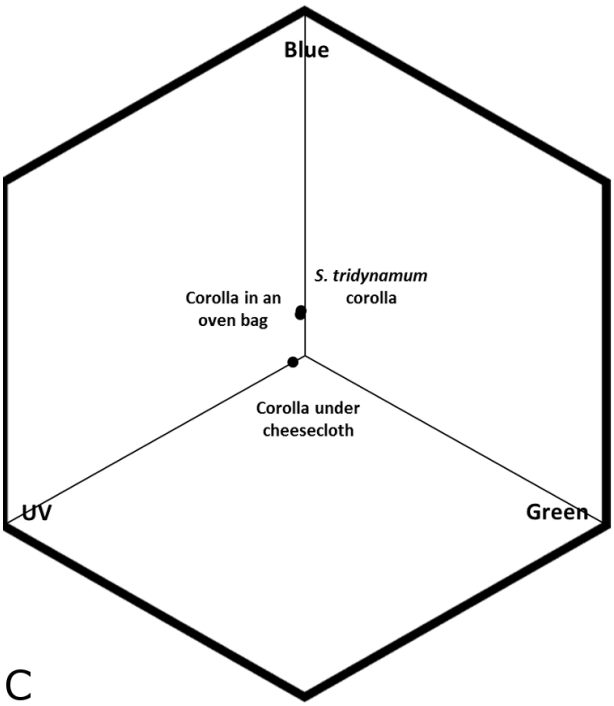


Figure S1. (A) The reflectance spectra of the foraging arena background and of the three types of *Solanum houstonii* flowers (Sham, Bag, and Cloth flowers) used in experiment 2. For spectra taken of flowers, spectra were made from the peripheral tissue and not the yellowish central tissue. (B) The irradiance of the fluorescent lights illuminating the foraging arena. The reflectance of corollas under cheesecloth was comparable to corollas not covered by anything. Cheesecloth increased reflectance by c. 10.7% relative to the foraging arena background at 400-700nm; below 400nm the difference became great (up

to c. 40%): however, the fluorescent light sources did not produce light below 400nm. (C) The loci in *Bombus impatiens* colour space of Sham, Bag, and Cloth flowers against the foraging arena background. Sham and Bag flowers resembled each other, as did Cloth flowers and the background.

Figure S2

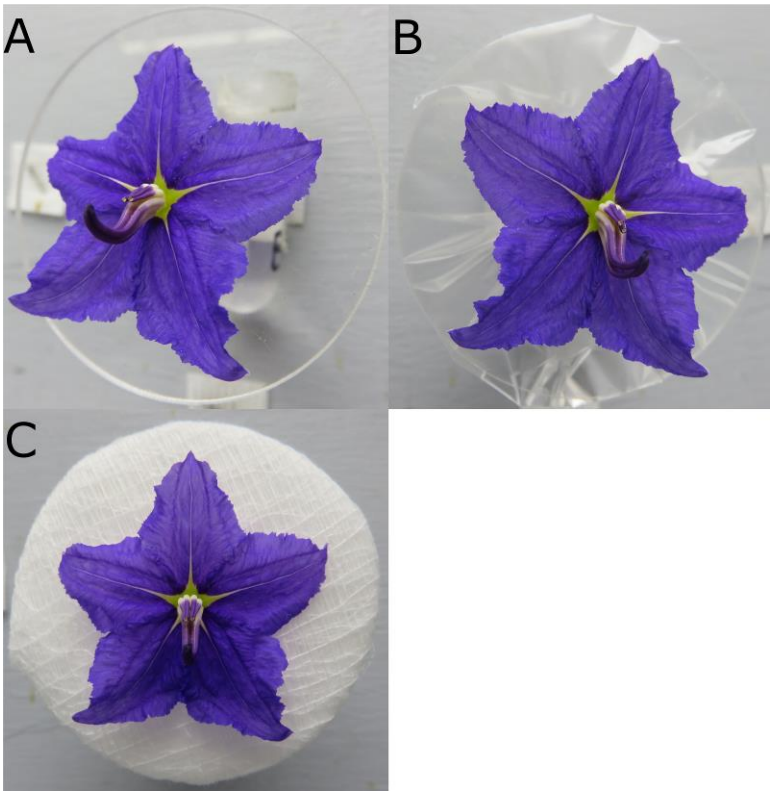


Figure S2. Sham *Solanum houstonii* flowers set against (A) no background, (B) an oven bag background, and (C) a cheesecloth background. Sham flowers were constructed by hot-gluing anthers to corolla, as a control for cutting of floral structures in related experiments.

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), oven bag (Bag), or no supplementary background (Sham). We found no significant differences in the proportion of approaches or landings (Fig. S3: Differences in pairwise posterior probabilities: approaches, Sham versus Bag, *PP* = 0.682; Sham versus Cloth, *PP* = 0.791; Bag versus Cloth, *PP* = 0.614; landings, Sham versus Bag, *PP* = 0.778; Sham versus Cloth, *PP* = 0.719; Bag versus Cloth, *PP* = 0.571).

Figure S3

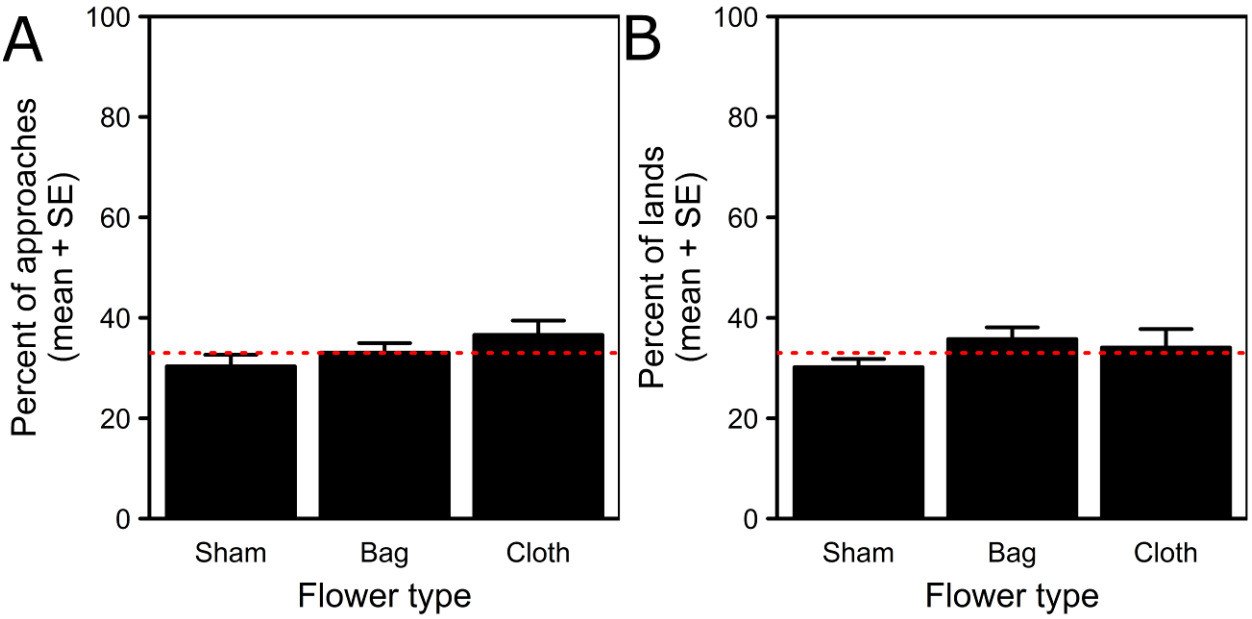


Figure S3. Percentage of responses (+SE) by initially-naïve bumble bees (*Bombus impatiens*) to sham flowers (*Solanum houstonii*) set against an oven bag (Bag flowers), cheesecloth (Cloth flowers), or no background (Sham flowers). (A) Approaches and (B)

landings. $N = 7$ bees. Dashed line at 33% indicates random expectation for an assay with three choices.

Long term memory of anther chemistry

In order 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 hours later. Twelve of 16 bees were successfully retested. During testing we presented the bee with unrewarding surrogate flowers arranged in a 3 x 3 grid without a central flower (eight total flowers), with pentane control and anther extract treated flowers alternated by position.

Experienced bees tested 24 hours 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 (Fig. S4: paired *t*-test: extract versus pentane, $t_{11} = 5.319$, $P < 0.0003$). Furthermore, the strength of this preference did not differ significantly from the bees' initial learned preference, 24 hours prior (Fig. S4; Welch two sample *t*-test: long term versus short term, $t_{21.97} = 0.920$, $P = 0.368$).

Figure S4

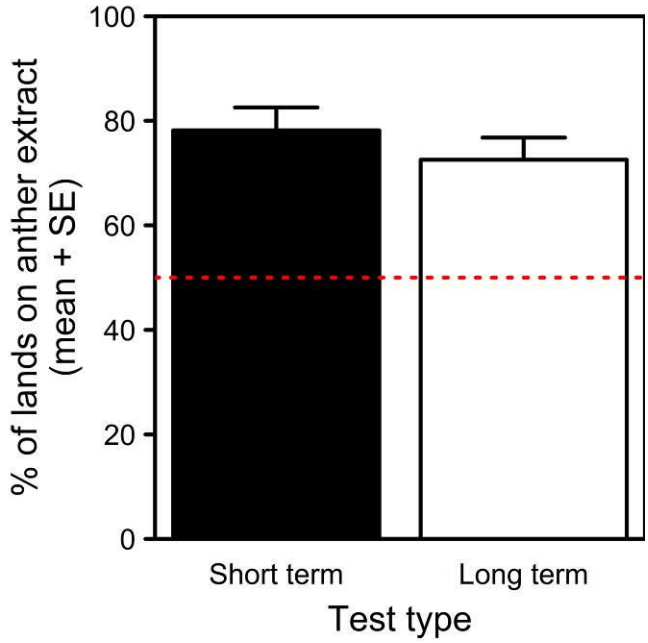


Figure S4. Mean percentage of landings (+SE) by *Bombus impatiens* on anther extract-treated surrogates (versus pure pentane treated surrogates); surrogates constructed from *Solanum houstonii* flowers and foam. Data shown for bees' short and long term retention tests. *N* = 16,12 bees for short and long term tests, respectively. Dashed line at 50% indicates random expectation for an assay with two choices.

APPENDIX D

CONCEALED FLORAL REWARDS AND THE ROLE OF EXPERIENCE IN FLORAL SONICATION BY BEES

FULL TITLE: Concealed floral rewards and the role of experience in floral sonication by bees

Avery L. Russell [a, *], Anne S. Leonard [c], Heather D. Gillette [b], Daniel R. Papaj [b]

[a] Graduate Interdisciplinary Program in Entomology and Insect Science, University of Arizona, Tucson, AZ, U.S.A.

[b] Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, U.S.A.

[c] Department of Biology, University of Nevada, Reno, NV, U.S.A.

*Corresponding author

This manuscript has been published:

Russell, A.L., Leonard, A.S., Gillette, H.D., Papaj, D.R. 2016. Concealed floral rewards and the role of experience in floral sonication by bees. *Animal Behaviour*. 120: 83-91, doi: 10.1016/j.anbehav.2016.07.024

ABSTRACT

Pollinators frequently use complex motor routines to find and extract floral rewards. Studies of pollinators foraging for nectar rewards indicate these routines are typically learned, and that constraints associated with learning and memory give pollinators incentive to continue foraging on these flowers. However, plants offer rewards besides nectar, including pollen, lipids and essential oils. In particular, bees use a complex motor routine termed floral sonication to extract pollen, their primary source of protein, from the more than 6% of flowering plant species (>22 000 species) that conceal pollen rewards within tube-like poricidal anthers. If floral sonication requires learning, this pollen extraction behaviour could contribute to floral fidelity. However, no studies have quantified the effect of experience on flower handling for bees extracting pollen from poricidal species. We therefore examined the degree to which floral sonication behaviour was modified by experience. We found that the key elements of the sonication motor routine appeared in full-blown form in a flower-naïve bee's first visit to a flower. We additionally found consistent, albeit modest, effects of experience on certain aspects of sonication behaviour. The latency to sonicate slightly decreased with experience. Bees also adjusted the length and amplitude of their sonication buzzes in response to pollen receipt. We conclude that the role of experience in foraging for concealed pollen rewards is different from that reported for nectar rewards. We offer an alternative explanation for its function in sonication. Finally, we discuss alternative hypotheses for the function of poricidal anthers and for how pollen-bearing plants may ensure floral fidelity even in the absence of a significant impact of experience on pollen extraction behaviour.

Keywords:

Bombus impatiens, bumblebee, buzz pollination, concealed reward, experience, floral reward, floral sonication, learning, mutualism, pollen collection

HIGHLIGHTS

- Bees successfully extracted concealed pollen on their first floral visit.
- Bees showed a small but rapid decrease in latency to sonicate after landing.
- Change in flower handling time did not persist or depend on pollen receipt.
- Bees modified features of sonication buzzes in response to pollen receipt.
- Pollen concealment likely does not drive floral fidelity via cognitive constraints.

INTRODUCTION

Pollinators often use complex motor routines to find and extract floral nectar. For instance, bees may have to enter the flower in the correct way or use their head and legs in a coordinated effort to pry open a flower's corolla to get to the nectar (e.g. Laverty & Plowright 1988; Westerkamp 1999). Such nectar extraction motor routines are frequently learned (Chittka et al. 1999). Accordingly, pollinators may require dozens of trips to become proficient at extracting nectar from the flowers of a given plant species (e.g. Gould 1984; Heinrich 1984; Lewis 1993). Because plant species vary in floral

morphology, pollinators such as bees must learn novel nectar extraction routines each time they shift to a new plant species (e.g. Laverty 1994; Gegear & Laverty 1995). Cognitive constraints associated with learning, forgetting and relearning these motor routines are thought to discourage pollinators from switching back and forth among plant species (Lewis 1993; Gegear & Laverty 1995; Chittka et al. 1999). Pollinators exhibiting floral fidelity provide direct benefits to the plant in terms of reduced pollen wastage and foreign pollen interference (Waser 1986; Gegear & Laverty 1995). In short, floral morphology that requires pollinators to use learned motor routines to access rewards is proposed to be an evolved strategy by which plants promote effective pollination services (Plowright & Laverty 1984; Lewis 1993; Chittka et al. 1999).

Although nectar is a common floral reward, it is not the only one. Bees, which are among our most important pollinators, must also collect pollen, their primary source of protein and a particularly common floral reward (Simpson & Neff 1981; Kevan & Baker 1983; Nicolson & van Wyk 2011). At least 6% of angiosperm species offer only pollen as a reward. Most of these species conceal pollen within specialized tube-like poricidal anthers (>22 000 species across >80 families: Buchmann 1983; Buchmann, Jolles, & Kreibel, n.d.). Pollinators of these so-called poricidal flowers, nearly exclusively bees, must extract the pollen using a complex motor routine termed floral sonication (Pellmyr 1988; Buchmann & Cane 1989). Sonicating bees rapidly contract their indirect flight muscles, thereby generating powerful vibrations (King & Lengoc 1993). These vibrations are transmitted through the bee's clasped mandibles to the poricidal anthers, which causes the pollen to be expelled onto the bee's body where it can be collected (Michener

1962; Switzer et al. 2015). The successful extraction of pollen thus involves coordination of legs (for positioning and grooming), mandibles and indirect flight muscles.

Floral fidelity could be facilitated if extraction of the pollen reward must be learned and if cognitive constraints like those proposed for nectar extraction result in costs to switch between flower types. In contrast to the role of learning in nectar collection, its role in pollen collection has scarcely been examined. Because floral sonication involves the use and coordination of multiple different motor units, similar in respects to the action of complex learned nectar extraction routines, it is reasonable to ask whether sonication is also learned. Previous work addressing this question is limited and has yielded mixed results. Two studies suggested that sonication might be innate (King 1993; Morgan et al. 2016), as bees buzz within their first few visits, while a third (Laverty 1980) reported that bees take time initially to sonicate, as well as to sonicate flowers effectively.

To our knowledge, no studies have quantified the effect of experience on flower handling for bees extracting pollen from poricidal species. In this study we characterized the behaviour of bees as in their first visits to poricidal flowers and quantified the motor movements involved in floral sonication. Additionally, we examined the possible influence of experience and receipt of a pollen reward on floral sonication behaviour.

METHODS

Subjects

We used 76 workers from six colonies of *Bombus impatiens* in experiments conducted between December 2013 and June 2014. We purchased colonies from Koppert Biological Systems (Howell, MI, U.S.A.) or from Biobest USA, Inc. (Romulus, MI, U.S.A.). We used equal numbers of bees from at least two colonies for each experiment.

Colonies had access to ad libitum unscented 2 M sucrose solution and pulverized honeybee-collected pollen (Koppert Biological Systems) within the foraging arena. Two feeders dispensed sucrose solution via braided cotton wicks (6-inch Braided Cotton Rolls, Richmond Dental, http://www.richmonddental.net/) that extended into 40-dram vials through perforations made in the human-white lids (BioQuip Products, Inc., Compton, CA, U.S.A.). Pollen was presented using two custom-made feeders (Fig. 1a; Russell & Papaj 2016) consisting of human-white chenille fibres, glued to the inside walls of 40-dram vials (BioQuip Products, Inc.). Neither type of feeder was scented or coloured in addition to the natural scent or colour of the sucrose solution or pollen. Bumble bees did not sonicate while collecting pollen from chenille fibres: bees always scrabbled for the pollen (additionally, of bees naïve to pollen foraging that were observed on their first few visits to chenille feeders, none sonicated). To our knowledge, honeybee-collected pollen is not collected from *Solanum* species (honeybees cannot collect the pollen because they cannot sonicate the poricidal anthers; Buchmann 1983) and could not have been

harvested from *Solanum tridynamum*, the focal plant species in our study. This plant species is endemic to Mexico, whereas the honeybee-collected pollen we used was harvested within the midwestern United States.

We used freshly clipped flowers from eight *S. tridynamum* plants in experiments. This species offers only pollen rewards via poricidal anthers (nectar is completely absent from this species). To extract the pollen, bees must vibrate the anthers via sonication. Two *S. tridynamum* were purchased locally (Arizona-Sonora Desert Museum, Tucson, AZ, U.S.A.) and six plants were raised from seeds. Plants were fertilized weekly (Miracle Gro, Marysville, OH, U.S.A., nitrogen-phosphorus-potassium = 15-30-15) and grown under natural light in a greenhouse.

General Experimental Protocol

All testing took place in a foraging arena (L × W × H = 82 × 60 × 60 cm) painted grey on floor and sides to provide a neutral background. To identify naïve bees suitable for testing, we allowed one to four flower-naïve workers into the arena simultaneously. When a flower-naïve bee landed on a flower in a test array, we removed the others from the arena immediately with vials. We always tested individual bees that had prior experience with *S. tridynamum* (see experiment 2) in the absence of other bees, to prevent social influences (Grüter & Leadbeater 2014). Specifically, bees (experiment 2) on their second and third day of testing were tested individually (in the absence of other bees): thus any changes in behaviour across floral visits would be intrinsic to the bees in their

response to the flower, and not the result of having other bees present or removed from the foraging arena. A bee was allowed to make a predetermined maximum number of visits (varying across subexperiments), after which we turned off the lights above the foraging arena, causing the bee to stop foraging, and collected the bee after 5 min. For experiment 1 we also ended a trial if a bee did not approach or land on a flower for 5 min. Upon completion of an assay, we froze and stored the bee at -18 °C.

In assays, freshly clipped flowers were horizontally displayed (the usual orientation of the flowers on the plants themselves) on custom-built water tubes, mounted on a foam block that matched the foraging arena background (Fig. 1). A single flower was made available to a test bee at any given time and each bee received a fresh, unused flower in each trial.

Behavioural Assays

Video for all tests was captured at 30 frames/s high definition with a digital camcorder (Canon VIXIA HF R400) suspended 2 cm from the flower (field of view was 5 cm centred on the flower). Audio was input to the camcorder at 3 ms sampling intervals using an external microphone (33-3013 Lavaliere Microphone, RadioShack, Ft Worth, TX, U.S.A.) suspended 2 cm from the flower. Video was analysed frame by frame using Avidemux software (fixounet@free.fr); audio was analysed using Audition 2.0 (Adobe Systems Inc., San Jose, CA, U.S.A.).

We recorded two behaviours: landing and sonication (buzzes) (Table 1). ‘Landings’ were categorized as ‘corolla landings’ and ‘anther landings’. Corolla and anther landings were defined as the bee touching the flower's corolla or anther, respectively, with at least three of the first four legs in the same video frame. Either type of landing marked the beginning of a ‘visit’. The end of a visit was defined as the first video frame in which the bee no longer contacted the flower with its legs. After landing, bees that placed their mandibles or the tarsi of their forelegs on the anther were noted as having located the anthers. We identified digitally recorded ‘sonications’ (a total of 4 186 buzzes; mean per bee ± SE: 380.5 ± 36.7), which only occurred after landing, by their sound, and which are distinct from flight buzzes and related sounds (A. Russell, personal observation). The location of buzzes (anther, corolla or off-flower) was recorded in terms of where the bee's mandibles were clamped at the time of the buzz (5.7% and 0.07% were delivered to the corolla and off-flower, respectively). We also recorded the duration of buzzes. We termed an ‘acceptance’ to be a visit that involved at least one sonication.

Additionally, we extracted the average amplitude of each buzz using a custom-built script (written by Callin Switzer in R v.3.2.0, R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). To allow the script to identify buzzes, we manually determined start and end time stamps for the script to read. The script then split each buzz into 512 sampling windows and created an average ‘volume value’ for each buzz. Volume value is a proxy for amplitude, based on bitrate provided by digitally recorded audio, which scales linearly, in contrast to the more commonly used decibel, which scales logarithmically. We used these volume values in analyses, hereafter referred

to as ‘amplitude’.

We analysed whether buzz length and amplitude were influenced by experience, because buzzes vary in these two attributes and are the key components of the extraction behaviour that determine whether the bee removes pollen (e.g. De Luca et al. 2013). Some studies (but not all, Burkart et al. 2011; Nunes-Silva et al. 2013, the latter being the only study on *B. impatiens*) that have tested for a relationship between body size and buzz length and amplitude have reported a correlation with buzz length (De Luca et al. 2014) or amplitude (De Luca et al. 2013). We therefore tested whether body size differed between treatments and whether buzz length and amplitude were significantly correlated with body size. We found that body size did not differ between treatments and buzz length and amplitude did not correlate significantly with body size (see Supplementary Material).

Behaviours Recorded Only for Ethograms

To construct ethograms, we recorded four additional behaviours: approaches, antennal contact, probing for nectar and bites (Table 1). An ‘approach’ was defined as the bee hovering within 3 cm of the flower. ‘Antennal contact’ was defined as the bee touching floral tissue for any duration with the terminal antennomer of either antenna. A ‘nectar-probe’ was defined as the bee extending and thrusting its tongue against any part of the corolla or anther with a distinctive whole-body bobbing action. The start of a ‘bite’ was defined as the first frame in which the mandibles were observed clamping onto the floral

tissue. For each bite on floral tissue where the tips of the bee's mandibles were visible, we measured the duration of the bite. The end of a bite was defined as the first frame in which the mandibles no longer enclosed floral tissue. The only other occasions where we observed opening or closing of the mandibles were when a bee extended its proboscis during grooming. We did not count these as part of a sonication motor sequence.

We constructed two ethograms. In one ethogram we report the elements up to and including the first buzz or until bees left the flower (see Results, Fig. 2). We used 48 bees visiting rewarding flowers for this analysis. The data for these visits were from 18 bees in experiment 1, 12 bees from experiment 2, and 18 additional bees that were treated identically to the rewarding treatment in experiment 1 (but not part of experiment 1 or 2). In the other ethogram we examined the functional elements of the floral sonication motor routine, including antennal contact, the buzz, the bite, and whether these elements were coincident (see Results, Fig. 3). For this analysis we examined the earliest anther buzz or bite that each bee performed on its first visit, for which the mandibles and antennae were clearly visible. We used 48 bees for this analysis, but discarded four of these bees that did not buzz on their first visit. We uncovered additional minor variation in the floral sonication motor routine when we examined the first 85 analysable buzzes of six of the undiscarded bees (Supplementary Table S1).

Experiment 1: Role of Experience and Receipt of Pollen

Here we sought to determine whether foraging behaviour changes with experience and

with pollen availability. This experiment used 36 bees from four colonies.

To create unrewarding flowers, we applied drops of glue (Elmer’s Glue All, Elmer's Products, Inc., Westerville, OH, U.S.A.) to the tip of each poricidal anther with a clean toothpick and allowed the glue to dry for 5 min. This action sealed the anther pore, preventing pollen release. We verified with a dissecting microscope that anther pores were closed. If bees broke open anthers during an experiment (usually at the ventral base of the locules) and thus released pollen, we discarded all observations post pollen release (we included observations prior to pollen release in analyses).

To control for the effects of glue scent we applied drops of glue to the distal sides of each anther for all rewarding flowers, without blocking the pores. Glue was allowed to dry for 5 min before flowers were used in experiments.

One group of naïve bees was presented with a rewarding flower and another group was presented with an unrewarding flower in this experiment. We allowed bees to make up to 10 acceptances on their particular flower, always in a single foraging bout. However, we report and analyse results only from the first six, because most bees did not complete all 10 (bees that dropped out earlier showed the same qualitative pattern as bees that dropped out later). We systematically alternated treatments to control for effects of time and day on behaviour.

A follow-up experiment comparing use of a single flower across all visits for a single bee

versus using multiple flowers confirmed that using only a single flower did not affect bee behaviour (see Supplementary Material).

Experiment 2: Long-term Retention of Behavioural Changes

Here we sought to determine whether any changes in pollen collection behaviour persist for days. We allowed each naïve bee two consecutive acceptances of a fresh flower on the first day, three consecutive acceptances of a fresh flower 24 h later, and three consecutive acceptances of a fresh flower 48 h after their first floral experience. Consecutive acceptances were always made within the same foraging bout. Five minutes after completing their first two acceptances bees were labelled with individually numbered plastic coloured tags (The Bee Works, Inc., Oro-Medonte, ON, Canada) attached by superglue to the dorsum of the thorax and returned to the colony box. This experiment used 12 bees from two colonies. We discarded two bees that died before completing the full experiment.

Data Analyses

All data were analysed using R v.3.2.0 (R Development Core Team). For experiment 1, to determine whether there was an effect of experience and treatment on the buzz latency across all six floral visits, we applied a learning curve to each bee's data and analysed the estimated parameters. We used a Wright's cumulative average model (Martin, n.d.). The model takes the form $Y = aX^b$, where Y is the cumulative buzz latency (measured in

seconds) per floral visit, *X* is the cumulative number of floral visits, *a* is the estimated buzz latency for the first floral visit, and *b* is the slope of the function in log–log space. To improve fit, we discarded data from bees that completed fewer than three visits (a total of 8 bees; 5 from the unrewarding treatment, 3 from the rewarding treatment). The fit of the model was very good: the mean coefficient of determination was high (rewarding: 0.79 ± 0.09; unrewarding: 0.85 ± 0.07), and as an additional check, we analysed whether the estimated parameter *a* differed significantly from the actual *a* for each treatment (it did not: see Supplementary Material).

To determine whether the effect of experience (i.e. *a* and *b*) differed between treatments, we used *t* tests if assumptions of normality and equal variance were met (using Shapiro–Wilk and *F* tests, respectively, in the 'mgcv' package: Wood, 2015). Otherwise, we used Wilcoxon signed-ranks tests using the 'wilcox.test()' function in R. Likewise, to determine whether there was an effect of experience at all in each treatment, we used one-sample *t* tests or Wilcoxon signed-ranks tests to determine whether estimated parameters differed from zero.

We also used a Wright's cumulative average model to determine whether there was an effect of experience on the anther discovery latency across floral visits for the rewarding treatment, following the techniques described above. Data from two bees that had completed fewer than three visits were discarded. Because the model could only utilize nonzero numbers and the anther discovery latency was frequently zero (i.e. bees landing on the anthers), we added 0.1 s to the anther discovery latency of each visit.

In addition, we examined the difference between the first and the second visit for the rewarding treatment for all 18 bees, as the change in buzz latency was greatest between the first two visits. We used Wilcoxon signed-ranks tests to compare latency across the first and second floral visit for the rewarding treatment. We ran Wilcoxon signed-ranks tests on the variable 'buzz latency' (measured in seconds) with 'first floral visit' or 'second floral visit' as matched samples.

For experiment 2 we used repeated measures MANOVA to determine whether the buzz latency drop (difference in the buzz latency from the first to the second floral visit) persisted across days (difference in the buzz latency from the first to the second floral visit on each of 3 consecutive days). We ran this multivariate test (Wilks' λ distribution) using the 'Anova()' function in the 'car' package (Fox & Weisberg 2011).

For experiment 1 we used linear mixed-effects models (LMM) to determine whether there was an effect of treatment and experience (the 'buzz number', not the visit number) on buzz length or buzz amplitude. We performed two LMMs: one for the response variable buzz length, one for the response variable buzz amplitude. We log transformed these variables to normalize the residuals. LMMs were specified via the 'lme()' function, in the 'lme4' package (Bates et al. 2015).

For these mixed models, we specified buzz length or buzz amplitude as the response variable, treatment ('rewarding' or 'unrewarding') and buzz number (the first 100 buzzes

for each initially flower-naïve bee) as fixed factors, with buzz number also included as a repeated measures factor within the random factor BeeID. To examine the possible significance of an interaction between buzz number and treatment, results were first examined using type III sums of squares via the 'Anova()' function. Because the interaction was not significant, we report results with type II Wald chi-square tests via the 'Anova()' function. For each analysis, we performed two rounds of backward elimination (as described in Fox 2015).

To determine whether there was a trade-off between buzz length and buzz amplitude, we tested for an association between paired samples of buzz amplitude and buzz length for each treatment separately, using the 'cor.test()' function in R.

RESULTS

The Basic Components of the Sonication Motor Routine Are Strongly Stereotyped

The behaviour of flower-naïve bumblebee workers on their first floral visit to *Solanum* flowers consisted of clearly definable components arranged in a predictable sequence (Figs 2, 3). First, the bee approached the flower (Fig. 2a,e). During the approach, bees contacted the flower with their antennae prior to landing 94% of the time (of the 35 bees where the position of the antennae during landing could be confirmed; Fig. 2b), consistent with the behaviour of bees approaching artificial flowers (Lunau 1992;

Evangelista et al. 2010). In the vast majority of cases (92% of 48 bees), a previously flower-naïve bee buzzed on its first floral visit (Fig. 2d,e). Antennal contact with floral tissue preceded buzzing 100% (of 48 bees) of the time (Fig. 3a). Of all bees, 13% (of 48 bees) attempted to probe for nectar (despite nectar not being produced by the flowers of this species) before sonicating thereafter.

The major transitions did not change substantially from the first to second floral visit (Fig. 2e versus 2f). Immediately prior to making their second landing on a flower, bees contacted the flower with their antennae 95% of the time (of 43 bees where the position of the antennae during landing could be confirmed). In all but one case (98% of 47 bees), bees buzzed on their second floral visit (Fig. 2f). Of the bees that buzzed, 81% (of 47 bees) buzzed the anthers first, and the remaining bees eventually buzzed the anthers as well (Fig. 2f). No bees attempted to probe for nectar before sonicating on their second visit.

We also observed that components of the sonication routine were coordinated in time and space, even in the very first floral visit. For example, for pollen to be extracted, the bee must buzz while biting the anthers. The location of the buzz is important, because only the anthers hold the pollen. The sequence is important, because buzzing generates powerful vibrations that eject pollen (King & Lengoc 1993), while biting allows bees to anchor themselves and most effectively transmit the buzz vibrations to the anthers (King & Buchmann 2003). Buzzes not coincident with bites did not result in perceptible amounts of pollen being released; bees that buzzed without biting occasionally ejected

themselves from flowers due to the force of the vibrations (A. Russell, personal observation). However, most bees showed a fully functional sonication motor routine even in their very first floral visit (Figs 2, 3). Of the bees that buzzed on their first floral visit, 89% (of 44 bees) buzzed the anthers first (Fig. 2e). After bees bit the anthers, they almost always buzzed (95% of 44 bees; Fig. 3d–i). Most sonications (80% of 44) were bounded by a single bite and nearly all sonication events (98% of 44) were at least coincident with a bite (Fig. 3d–i).

Amplitude and Length of Buzzes Varies with Pollen Availability and Experience

Bees that encountered and buzzed flowers that could not release pollen had significantly shorter and louder buzzes than bees that encountered and buzzed flowers that released pollen (Fig. 4a; LMM for buzz length: treatment effect: $\chi^2 = 7.4627$, $P < 0.007$; Fig. 4b; LMM for buzz amplitude: treatment effect: $\chi^2 = 16.624$, $P < 0.0001$). These differences correspond to a 26.1% difference in mean duration and a 2.97 dB difference (a 146.8% difference in mean amplitude and a 198.2% difference in power) in sonications for bees buzzing rewarding versus unrewarding flowers.

In addition, for both treatments, the length of buzzes was significantly positively correlated with the amplitude of buzzes: e.g. longer buzzes were also louder (Pearson's correlation: rewarding: $r = 0.278$, $t_{598} = 7.0661$, $N = 6$, $P < 0.0001$; unrewarding: $r = 0.240$, $t_{698} = 6.5347$, $N = 7$, $P < 0.0001$). These results indicate that the changes in buzz length and amplitude in response to pollen receipt were not the result of a trade-off

between these two characteristics.

For both treatments, bees increased the length and amplitude of their buzzes with experience (LMM for buzz length: buzz number effect: $\chi^2 = 4.5921$, $P < 0.033$; Fig. 4a; LMM for buzz amplitude: buzz number effect: $\chi^2 = 4.5608$, $P < 0.033$; Fig. 4b). There was no significant interaction between experience and treatment for either length or amplitude of buzzes (LMM for buzz length: buzz number*treatment effect: $\chi^2 = 0.1126$, $P = 0.737$; LMM for buzz amplitude: buzz number*treatment effect: $\chi^2 = 0.276$, $P = 0.600$; Fig. 4a).

Latency to Sonicate the Flower Changes with Experience

Naïve bees did not sonicate immediately after landing on a rewarding flower. This initial latency was highly variable across bees, but over subsequent visits the latency to sonicate dropped significantly (*t* tests: difference from zero of the learning curve's slope (parameter *b*): $t_{14} = -6.4125$, $P < 0.0001$; difference from zero of the learning curve's intercept (parameter *a*): $t_{14} = 4.754$, $P < 0.0004$; Fig. 5a). In fact, from the first to second visit, the latency to sonicate dropped significantly (latency, first visit versus second visit, Wilcoxon two-sample test: $W = 652$, $N = 18$ bees, $P < 0.0008$) and stays low.

However, naïve bees did not significantly improve their ability to find the anthers of a rewarding flower over the first six visits (*t* tests: difference from zero of the learning curve's slope (parameter *b*): $t_{14} = 0.7516$, $P = 0.464$; parameter *b* mean ± SE = 0.13 ±

0.18; difference from zero of the learning curve's intercept (parameter *a*): t_{14} = -1.4682, *P* = 0.163; parameter *a* mean ± SE = -0.23 ± 0.16; *N* = 16 bees), despite making a greater proportion of landings on the anthers on the second visit (Fig. 2e versus 2f).

In addition, the pattern of the latency drop was independent of pollen receipt: it did not differ significantly between bees accepting flowers that released or did not release pollen (Fig. 5a; *t* tests: learning curve's slope (parameter *b*), treatment effect: $t_{21.752}$ = -0.5202, *P* = 0.608; learning curve's intercept (parameter *a*): $t_{25.675}$ = 0.4146, *P* = 0.682).

Finally, the drop in the latency to sonicate did not persist across days: the magnitude of the latency drop from the first to the second floral visit did not differ across 3 successive days (MANOVA: $F_{2,8}$ = 0.0145, *P* = 0.986; Fig. 5b).

DISCUSSION

Floral fidelity by pollinators is thought to be, at least in part, a consequence of cognitive constraints associated with learning and recalling how to extract concealed floral rewards (Lewis 1993; Chittka et al. 1999; Gegear & Laverty 2005). Concealment of floral rewards has even been proposed as an evolved strategy by which plants maintain floral fidelity by pollinators (Lewis 1993). While pollinators such as bees, many flies and some butterflies collect both pollen and nectar (Kevan & Baker 1983; Nicolson & van Wyk 2011), the cognitive constraints associated with concealed rewards have only been

studied in the context of nectar rewards. However, cognitive constraints, and by extension, floral fidelity, as a result of reward concealment should not depend on whether the reward being concealed is pollen or nectar. Surprisingly, our findings suggest that cognitive constraints do depend on reward, with respect to sonication: bees visiting flowers with poricidal anthers displayed sonication behaviour that was fully expressed and highly effective in their very first floral visit. By contrast, nectar-foraging bees typically fail to find concealed nectar rewards altogether on their first few visits (Laverty 1980, 1994; Laverty & Plowright 1988). The effectiveness of floral sonication appeared to vary little over time, whereas the effectiveness of nectar extraction often changes substantially with experience (Laverty 1994).

While sonication behaviour clearly is spontaneously performed, it nevertheless showed a modest degree of plasticity. For instance, bees decreased the length and increased the amplitude of their sonication buzzes in response to pollen receipt. This result itself is not necessarily learning, but might suggest a capacity for pollen receipt to modify characteristics of sonication buzzes in response to the particular plant species being foraged from. Pollen receipt is known to modify aspects of behaviour other than sonication. For example, we found previously that bees rapidly adopt landing preferences for the particular buzz-pollinated plant species from which they have successfully collected pollen, and these preferences appear to involve learning of anther cues (Russell et al. 2015).

We additionally observed in the present study that bees modified how quickly they

sonicated anthers after landing. Nectar-foraging pollinators show a similar pattern, improving the speed with which they can discover floral nectar with experience (‘handling time’) (Lewis 1986; Woodward & Laverty 1992; Gegear & Laverty 1998). In fact, changes in handling time are thought to be a major cost of learning to forage for nectar on a novel flower type (Lewis 1993).

Does the change in the latency to sonicate reflect learning how to extract pollen from poricidal anthers? If this change in handling time were chiefly a result of learning to associate floral cues with the acquisition of a pollen reward (that is, associative learning; Giurfa 2007), bees that successfully extracted pollen should sonicate sooner in subsequent floral visits, relative to bees that did not extract pollen. However, in our experiments, bees reduced their handling time whether they were assigned to rewarding or unrewarding pollen treatments. The change in latency might still be associative learning, but might also be a form of nonassociative learning. Alternatively, it may not be not learning at all, but a priming of general motivation for collecting pollen.

Whether or not the drop in the latency to sonicate constitutes learning, we can still ask if the initial delay in extracting pollen, and any associated cognitive constraints in reducing the delay, affected foraging efficiency enough to promote floral fidelity. We believe it did not for two reasons. First, the initial delay was short, on the order of 3–6 s. This was a much smaller time cost than has been reported in nectar extraction handling time studies (Laverty 1994). Second, the delay was reduced rapidly, after a single visit. In contrast, bees often take dozens of visits to learn to efficiently locate concealed nectar (Woodward

& Laverty 1992; Laverty 1994; Gegear & Laverty 1995). Taking these two factors together, the observed pattern of change with experience seems unlikely to affect foraging efficiency and thus unlikely to mediate floral fidelity directly.

Even if cognitive constraints associated with extracting pollen from poricidal species are unlikely to lead to floral fidelity, poricidal anthers might benefit plants in other ways. For instance, concealment of pollen within poricidal anthers may protect pollen from abiotic factors, such as rain, fluctuations in humidity and ultraviolet radiation (Gottsberger & Silberbauer-Gottsberger 1988; Johnson & McCormick 2001; Edlund et al. 2004; Zhang et al. 2014). Poricidal morphology may also restrict the amount of pollen a forager can remove for its own use (Castellanos et al. 2006; Hargreaves et al. 2009). Poricidal anthers may pave the way to more efficient pollination in still other ways. Only a limited range of pollinators can collect pollen from poricidal anthers, specifically the 58% of bee species that sonicate (Buchmann 1983; Cardinale, Russell, & Buchmann, n.d.). Such restriction could facilitate the evolution of floral adaptations tailored to those pollinators that lead to enhanced pollination success (Anderson et al. 2009; Newman et al. 2014). For instance, many poricidal species have evolved stamens divided into ‘feeding’ and ‘pollinating’ functions (i.e. heteranthery), which further reduces pollen wastage (Vallejo-Marín et al. 2010; Li et al. 2015).

An important question remains: in the absence of cognitive constraints on pollen extraction behaviour, how does a pollen-rewarding plant ensure that a bee shows fidelity and thereby transfers pollen to conspecifics? Possibly, cognitive constraints occur in other components of the foraging sequence. For instance, with experience, bees can

increase the amount of pollen they transport (Raine & Chittka 2007). Furthermore, as mentioned above, bumblebees form strong, durable landing preferences for pollen-only plant species with which they have experience (Russell et al. 2015); bees are likely learning visual and olfactory cues to identify plant species (Muth et al. 2015, 2016; Russell et al. 2015; but see also Arenas & Farina 2012; Nicholls & de Ibarra 2014, which use nectar-infused pollen). It is conceivable that the need to learn such cues imposes cognitive constraints that promote floral fidelity. Testing this hypothesis would involve assessing costs associated with switching during pollen collection from one plant species to another, as has been done in the context of nectar collection (Lewis 1986; Gegear & Laverty 1995, 1998). If learning of these floral cues drives floral fidelity, then bees should show significant losses in foraging efficiency when switching back and forth between species. Lastly, while floral fidelity is thought to be a common mechanism resulting in the conspecific transport of pollen, further work will be required to investigate whether bees foraging for pollen exhibit floral fidelity in a manner analogous to bees foraging for nectar (Waser 1986; Gegear & Laverty 1995; Chittka et al. 1999).

Although sonication behaviour appears not to be learned and thus cannot itself drive floral fidelity via cognitive constraints, as proposed for nectar collection behaviour, it may still play an important role in maintaining floral fidelity. Because sonication behaviour is immediately expressed in full-blown form, it probably facilitates learning of cues related to finding and recognizing plant species with poricidal anthers (>22 000 species, or more than 6% of angiosperm species). Sonicating bees immediately receive pollen, which in turn immediately reinforces responses to floral signals that identify a

rewarding plant species. In this way, the congenital expression of sonication behaviour could make that form of learning a more important driver of floral fidelity than if sonication behaviour itself was learned.

ACKNOWLEDGMENTS

We are grateful to Mark Borgstrom and Mohammad Torabi for aid with statistical analyses, to Abreeza Zegeer for greenhouse care, to Daniel Rojas, Cynthia Trefois and Eleni Moschonas for transcribing videos and measuring bees, to China Rae Newman for assistance in running experimental trials, and to Callin Switzer for the R script to analyse sonication amplitude. This work was supported by the National Science Foundation (IOS-1257762 to A.S. Leonard, S. L. Buchmann and D. R. Papaj) and an Entomology and Insect Science Graduate Student Research Support Award.

REFERENCES

Anderson, B., Alexandersson, R., & Johnson, S. D. (2009). Evolution and coexistence of pollination ecotypes in an African *Gladiolus* (Iridaceae). *Evolution*, 64(4), 960-972. http://dx.doi.org/10.1111/j.1558-5646.2009.00880.x

Anderson, B., Ros, P., Wiese, T. J., & Ellis, A. G. (2014). Intraspecific divergence and convergence of floral tube length in specialized pollination interactions. *Proceedings of the Royal Society B: Biological Sciences*, 281, 20141420. http://dx.doi.org/10.1098/rspb.2014.1420

Arenas, A., & Farina, W.M. (2014). Bias to pollen odors is affected by early exposure and foraging experience. *Journal of Insect Physiology*, 66, 28-36.

Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67(1), 1-48. doi:10.18637/jss.v067.i01.

Bell, G. (1986). The evolution of empty flowers. *Journal of Theoretical Biology*, 118, 253-258.

Buchmann, S. L. (1983). Buzz pollination in angiosperms. In C. E. Jones & R. J. Little (Eds.), *Handbook of experimental pollination biology* (pp. 73–113). New York, NY: Van Nostrand Reinhold.

Buchmann, S. L., & Buchmann, M. D. (1981). Anthecology of *Mouriri myrtilloides* (Melastomataceae Memcyleae), an oil flower in Panama. *Biotropica*, 13(2), 7-24.

Buchmann, S. L., & Cane, J. H. (1989). Bees assess pollen returns while sonicating *Solanum* flowers. *Oecologia*, 81, 289-294.

Buchmann, S.L., Jolles, D.D., Kreibel, R. (n.d.). *Angiosperms get buzzed many times, independently*. Manuscript in preparation.

Burkart, A., Lunau, K., & Schlindwein, C. (2011). Comparative bioacoustical studies on flight and buzzing of neotropical bees. *Journal of Pollination Ecology*, 6(16), 118-124.

Castellanos, M. C., Wilson, P., Keller, S. J., Wolfe, A. D., & Thomson, J. D. (2006). Anther evolution: pollen presentation strategies when pollinators differ. *American Naturalist*, 167, 2, 288-296.

Chittka, L., Thomson, J. D., & Waser, N. M. (1999). Flower constancy, insect psychology and plant evolution. *Naturwissenchaften*, 86, 361-377.

De Luca, P. A., Bussière, L. F., Souto-Vilaros, D., Goulson, D., Mason, A. C., & Vallejo-Marín, M. (2013). Variability in bumblebee pollination buzzes affects the quantity of pollen released from flowers. *Oecologia*, *172*(3), 805–816. http://dx.doi.org/10.1007/s00442-012-2535-1

De Luca, P.A., Cox, D.A., & Vallejo-Marín, M. (2014). Comparison of pollination and defensive buzzes in bumblebees indicates species-specific and context-dependent vibration. *Naturwissenschaften*, *101*(4), 331–338. http://dx.doi.org/10.1007/s00114-014-1161-7

Edlund, A. F., Swanson, R., & Preuss, D. (2004). Pollen and stigma structure and function: the role of diversity in pollination. *Plant Cell*, 16(Suppl.), S84-S97. www.plantcell.org/cgi/doi/10.1105/tpc.015800

Evangelista, C., Kraft, P., Dacke, M., Reinhard, J., & Srinivasan, M.V. (2010). The

moment before touchdown: landing manoeuvres of the honeybee *Apis mellifera*. *Journal of Experimental Biology, 213*, 262-270.

Fox, J. (2015). *Applied regression analysis and generalized linear models* (3rd ed.). London, U.K.: Sage.

Fox, J., & Weisberg. S. (2011). *An R companion to applied regression* (2nd ed.). Thousand Oaks CA: Sage. http://socserv.socsci.mcmaster.ca/jfox/Books/Companion

Freitas, L., & Sazima, M. (2003). Floral biology and pollination mechanisms in two viola species: from nectar to pollen flowers? *Annals of Botany*, 91, 311-317. http://dx.doi.org/10.1093/aob/mcg025

Gegear, R. J., & Laverty, T. M. (1995). Effect of flower complexity on relearning flower-handling skills in bumble bees. *Canadian Journal of Zoology*, 73, 2052-2058.

Gegear, R. J., & Laverty, T. M. (1998). How many flower types can bumble bees work at the same time? *Canadian Journal of Zoology*, 76, 1358-1365.

Gegear, R. J., & Laverty, T. M. (2005). Flower constancy in bumblebees: a test of the trait variability hypothesis. *Animal Behaviour*, 69, 939-949. http://dx.doi.org/10.1016/j.anbehav.2004.06.029

Giurfa, M. (2007). Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. *Journal of Comparative Physiology, 193*(8), 1-24. http://dx.doi.org/10.1007/s00359-007-0235-9

Gottsberger, G., & Silberbauer-Gottsberger, I. (1988). Evolution of flower structures and pollination in neotropical *Cassiinae* (Caesalpiniaceae) species. *Phyton*, 28(2), 293-320.

Gould, J. L. (1984). Natural history of honey bee learning. In P. Marler & H. S. Terrace (Eds.), *The biology of learning* (pp. 149-180). Berlin, Germany: Springer-Verlag.

Grüter, C., & Leadbeater, L. (2014). Insights from insects about adaptive social information use. *Trends in Ecology & Evolution*, 29(3), 177-184. http://dx.doi.org/10.1016/j.tree.2014.01.004

Hargreaves, A. L., Harder, L. D., & Johnson, S. D. (2009). Consumptive emasculation: the ecological and evolutionary consequences of pollen theft. *Biological Reviews*, 84, 259-276. http://dx.doi.org/10.1111/j.1469-185X.2008.00074.x

Heinrich, B. (1984). Learning in invertebrates. In P. Marler & H.S. Terrace (Eds.), *The biology of learning* (pp. 135-148). Berlin, Germany: Springer-Verlag.

Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical Journal*, 50(3), 346-363.

Johnson, S. A., & McCormick, S. (2001). Pollen germinates precociously in the anthers of raring-to-go, an *Arabidopsis* gametophytic mutant. *Plant Physiology*, 126, 685-695.

Kevan, P. G. (1983). Insects as flower visitors and pollinators. *Annual Review of Entomology*, 28, 407-453.

King, M.J., & Buchmann, S.L. (2003). Floral sonication by bees: mesosomal vibration by *Bombus* and *Xylocopa*, but not *Apis* (Hymenoptera: Apidae), ejects pollen from poricidal anthers. *Journal of the Kansas Entomological Society*, 76(2), 295–305.

King, M.J., & Lengoc, L. (1993). Vibratory pollen collection dynamics. *Transactions of the American Society of Agricultural Engineers*, 36, 135-140.

Laverty, T. M. (1980). The flower-visiting behavior of bumble bees: floral complexity and learning. *Canadian Journal of Zoology*, 58, 1324-1335.

Laverty, T. M. (1994). Bumble bee learning and flower morphology. *Animal Behaviour*, 47, 531-545.

Laverty, T. M., & Plowright, R. C. (1988). Flower handling by bumblebees: a comparison of specialists and generalists. *Animal Behaviour*, 36, 733-740.

Leonard, A.S., Dornhaus, A., & Papaj, D.R. (2012). Why are floral signals complex? An outline of functional hypotheses. In S. Patiny (Ed.), *Evolution of plant–pollinator relationships* (pp. 279-300). Cambridge, U.K.: Cambridge University Press.

Lewis, A.C. (1986). Memory constraints and flower choice in *Pieris rapae. Science*, 232, 863-865.

Lewis, A.C. (1993). Learning and the evolution of resources: pollinators and flower morphology. In D. R. Papaj & A. C. Lewis (Eds.), *Insect learning: Ecological and evolutionary perspectives* (pp. 219–242). New York, NY: Chapman & Hall.

Li, J.K., Song, Y.-P., Xu, H., Zhang, Y.-W., Zhu, J.-Y., & Tang, L.-L. (2015). High ratio of illegitimate visitation by small bees severely weakens the potential function of heteranthery. *Journal of Plant Ecology*, 8(2), 213-223. http://dx.doi.org/10.1093/jpe/rtv021

Lunau, K. (1991). Innate recognition of flowers by bumble bees: orientation of antennae to visual stamen signals. *Canadian Journal of Zoology*, 70, 2139-2144.

Martin, J. R. (n.d.). *What is a learning curve? Management and accounting web*.

http://maaw.info/LearningCurveSummary.htm (accessed May 2016).

Michener, C. D. (1962). An interesting method of pollen collection by bees with tubular flowers. *Revista de Biologica Tropical*, 10(2), 167-175.

Morgan, T., Whitehorn, P., Lye, G.C., Vallejo-Marin, M. (2016). Floral sonication is an innate behaviour in bumblebees that can be fine-tuned with experience in manipulating flowers. *Journal of Insect Behavior*, http://dx.doi.org/10.1007/s10905-016-9553-5

Muth, F., Papaj, D. R., & Leonard, A. S. (2015). Colour learning when foraging for nectar and pollen: bees learn two colours at once. *Biology Letters*, 11, 20150628. http://dx.doi.org/10.1098/rsbl.2015.0628

Muth, F., Papaj, D. R., & Leonard, A. S. (2016). Bees remember flowers for more than one reason: pollen mediates associative learning. *Animal Behaviour*, 111, 93-100. http://dx.doi.org/10.1016/j.anbehav.2015.09.029

Newman, E., Manning, J., & Anderson, B. (2014). Matching floral and pollinator traits through guild convergence and pollinator ecotype formation. *Annals of Botany*, 113, 373-384. http://dx.doi.org/10.1093/aob/mct203

Nicolson, S. W. (2011). Bee food: the chemistry and nutritional value of nectar, pollen and mixtures of the two. *African Zoology*, 46(2), 197-204. http://dx.doi.org/10.1080/15627020.2011.11407495

Nicholls, E., & Hempel de Ibarra, N. (2014). Bees associate colour cues with differences in pollen rewards. *Journal of Experimental Biology*, 217, 2783-2788. http://dx.doi.org /10.1242/jeb.106120

Nunes-Silva, P., Hnrcir, M., Shipp, L., Imperatriz-Fonseca, V.L., & Kevan, P.G. (2013). The behaviour of *Bombus impatiens* (Apidae, Bombini) on tomato (*Lycopersicon esculentum* Mill., Solanaceae) flowers: pollination and reward perception. *Journal of Pollination Ecology*, 11(5), 33-40.

Pélabon, C., Thöne, P., Hansen, T. F., & Armbruster, W. S. (2012). Signal honesty and cost of pollinator rewards in *Dalechampia scandens* (Euphorbiaceae). *Annals of Botany*, 109, 1331-1339. http://dx.doi.org/10.1093/aob/mcs091

Pellmyr, O. (1988). Bumble bees (Hymenoptera: Aipdae) assess pollen availability in *Anemonpsis macrophylla* (Ranunculaceae) through floral shape. *Annals of the Entomological Society of America*, 81, 792-797.

Plowright, R. C., & Laverty, T. M. (1984). The ecology and sociobiology of bumble bees. *Annual Review of Entomology*, 29, 175-199.

R Development Core Team. (2010). *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Raguso, R. A. (2004). Flowers as sensory billboards: progress towards an integrated understanding of floral advertisement. *Current Opinion in Plant Biology*, 7, 434-440. http://dx.doi.org/10.1016/j.pbi.2004.05.010

Raine, N.E., & Chittka, L. (2007). Pollen foraging: learning a complex motor skill by bumblebees (*Bombus terrestris*). *Naturwissenschaften*, 94, 459-464.

Renner, S. S. (1989). A survey of reproductive biology in neotropical Melastomataceae and Memecylaceae. *Annals of the Missouri Botanical Garden*, 76(2), 496-518.

Russell, A. L., Golden, R. E., Leonard, A. S., & Papaj, D. R. (2015). Bees learn preferences for plant species that offer only pollen as a reward. *Behavioral Ecology*, 27(3), 731–740. http://dx.doi.org/10.1093/beheco/arv213

Russell, A.L., & Papaj, D.R. (2016). Artificial pollen dispensing flowers and feeders for bee behaviour experiments. *Journal of Pollination Ecology*, 18(3), 13-22.

Simpson, B. B., & Neff, J. T. (1981). Floral rewards: alternatives to pollen and nectar. *Annals of the Missouri Botanical Garden*, 68(2), 301-322.

Switzer, C. M., Hogendoorn, K., Ravi, S., & Combes, S. A. (2015). Shakers and head bangers: differences in sonication behavior between Australian *Amegilla murrayensis* (blue-banded bees) and North American *Bombus impatiens* (bumblebees). *Arthropod-Plant Interactions, 10*(1), 1-8. http://dx.doi.org/10.1007/s11829-015-9407-7.

Vallejo-Marín, M., Da Silva, E.M., Sargent, R.D., & Barrett, S.C.H. (2010). Trait correlates and functional significance of heteranthery in flowering plants. *New Phytologist*, 188, 418-425. http://dx.doi.org/10.1111/j.1469-8137.2010.03430.x

Varassin, I. G., Penneys, D. S., & Michelangeli, F. A. (2008). Comparative anatomy and morphology of nectar-producing Melastomataceae. *Annals of Botany*, 102, 899-909. http://dx.doi.org/10.1093/aob/mcn180

Waser, N. M. (1986). Flower constancy: definition, cause and measurement. *American Naturalist*, 127(5), 593-603.

Westerkamp, C. (1999). Keel flowers of the Polygalaceae and Fabaceae: A functional comparison. *Botanical Journal of the Linnean Society*, 129, 207-221.

Wood, S. (2015). *Mixed GAM computation vehicle with GCV/AIC/REML smoothness estimation* (R package version 1.9-9). https://stat.ethz.ch/R-manual/R-devel/library/mgcv/html/mgcv-package.html (accessed December 2015)

Woodward, G. L., & Laverty, T. M. (1992). Recall of flower handling skills by bumble bees: a test of Darwin's interference hypothesis. *Animal Behaviour*, 44, 1045-1051.

Ye, X.-Q., Meng, J.-L., Zhao, Z.-G., Fan, B.-L., & Du, G.-Z. (2011). Optimal pollinator attraction strategies in *Trollius ranunculoides* Hemsl. (Ranunculaceae) at different altitudes: increased floral display or promotion of nectar output? *Plant Biology*, 13, 551-555.

Zhang, C., Yang, Y.-P., & Duan, Y.-W. (2014). Pollen sensitivity to ultraviolet-B (UVB) suggests floral structure evolution in alpine plants. *Scientific Reports*, 4, 4520. http://dx.doi.org/10.1038/srep04520.

FIGURE LEGENDS

Figure 1. (a) Chenille stem feeder loaded with honeybee-collected pollen (reproduced from Russell & Papaj, 2016). (b) Grey foam block used to mount water tubes, from which flowers were displayed. Each water tube held only a single flower. Flowers were displayed horizontally and glued into the water tube, ensuring that floral display was uniform across all treatments and experiments.

Figure 2. (a–d) Key phases in the first floral visit up until the first buzz or until the bee left the flower: (a) approaching the flower, (b) antennal contact with the flower indicated with red arrow, (c) landing indicated with red arrow, (d) buzzing anthers. Anthers indicated by the red letter 'A'; corolla indicated by the red letter 'C'. (e) Ethogram of the first floral visit. (f) Ethogram of the second floral visit. Arrows indicate the transition from one behavioural component to another. The transition frequency is indicated by both the number and thickness of the arrow. We calculated values by dividing the average number of transitions for a particular component by the total number of transitions derived from a behavioural element. Thus, transition frequencies reflect only the transitions from a given component to any other component (i.e. all transitions from a given component add up to one). We report data from the averaged response of the first and second visit sequence for 48 bees (one bee was discarded for the second sequence, as it made only a single visit).

Figure 3. (a–c) Key phases in the floral sonication motor routine: (a) antennal contact and clamping the mandibles on floral tissue, (b) sonicating; blur caused by vibration of wings during buzz indicated with red arrow, (c) releasing mandibles. (d–i) The six kinds of floral sonication motor routines observed in the earliest analysable sequence for each bee on its first floral visit: (d) bite begins before and ends after buzz, (e) a bite with no buzz, (f) a buzz with no bite, (g) buzz begins before bite and ends after bite, (h–i) buzz and bite are offset positively or negatively. Brackets indicate bite duration, stylized sonograms indicate buzz duration, and percentages indicate the relative frequency of each sequence. We analysed the earliest anther buzz or bite that each bee performed on its first visit, for which the mandibles and antennae were clearly visible (mean number of buzzes/bites to find an analysable sequence ± SE: 3.59 ± 0.52, N = 44 bees).

Figure 4. Mean ± SE (a) duration and (b) amplitude of sonications for the first 100 buzzes (rewarding treatment: N = 6 bees; unrewarding treatment: N = 7 bees). Although analyses were performed on log-transformed data, means and SEs are shown for the untransformed data.

Figure 5. (a) Mean ± SE latency to sonicate on the first six floral visits to a rewarding flower (N = 15 bees) and an unrewarding flower (N = 13 bees). (b) Mean ± SE latency to sonicate on rewarding flowers for the first three floral visits and across days (N = 10 bees).

FIGURES & TABLES

Table 1

Behaviours recorded

Behaviour	Description
Approach*	Hovering within 3 cm of a flower
Landing	Bee touching a flower's corolla or anther, with at least three of the first four legs in the same video frame
Visit end	The first video frame in which the bee no longer contacted the flower with its legs
Sonication (buzzes)	An attempt to extract pollen, which occurred only after landing: identified by their distinctive sound
Probing for nectar*	Extending and thrusting tongue against any part of the corolla or anther with a distinctive whole-body bobbing action
Antennal contact*	Touching floral tissue for any duration with the terminal antennomer of either antenna
Bite*	Clamping mandibles onto floral tissue: the start of a bite was defined as the first frame in which the mandibles were observed clamping onto floral tissue; the end of a bite was defined as the first frame in which the mandibles no longer enclosed floral tissue

*Only used to construct ethograms**.**

Figure 1

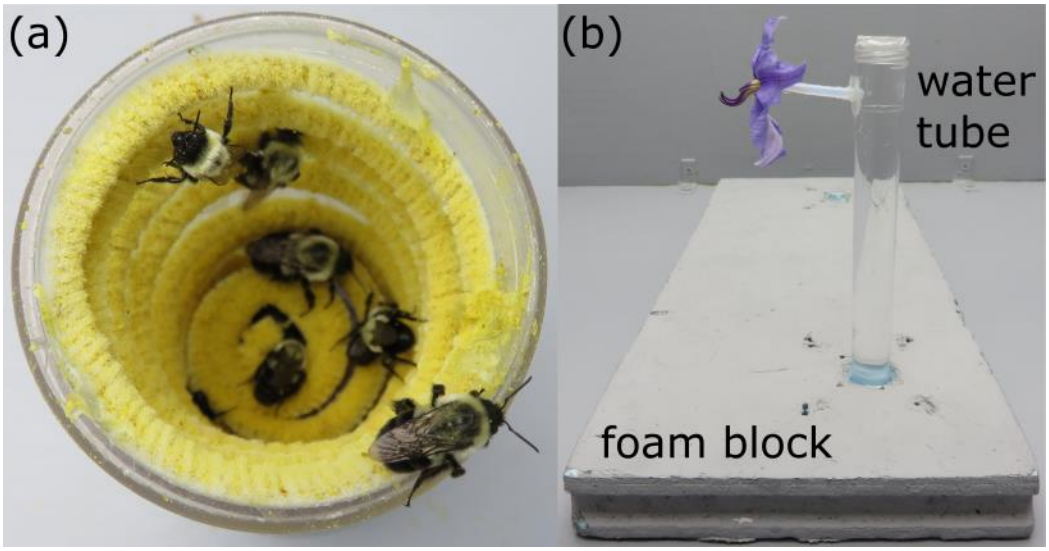


Figure 1. (a) Chenille stem feeder loaded with honeybee-collected pollen, reproduced from Russell & Papaj 2016. (b) Grey foam block used to mount water tubes, from which flowers were displayed. Each water tube held only a single flower. Flowers were displayed horizontally and glued into the water tube, ensuring that floral display was uniform across all treatments and experiments.

Figure 2

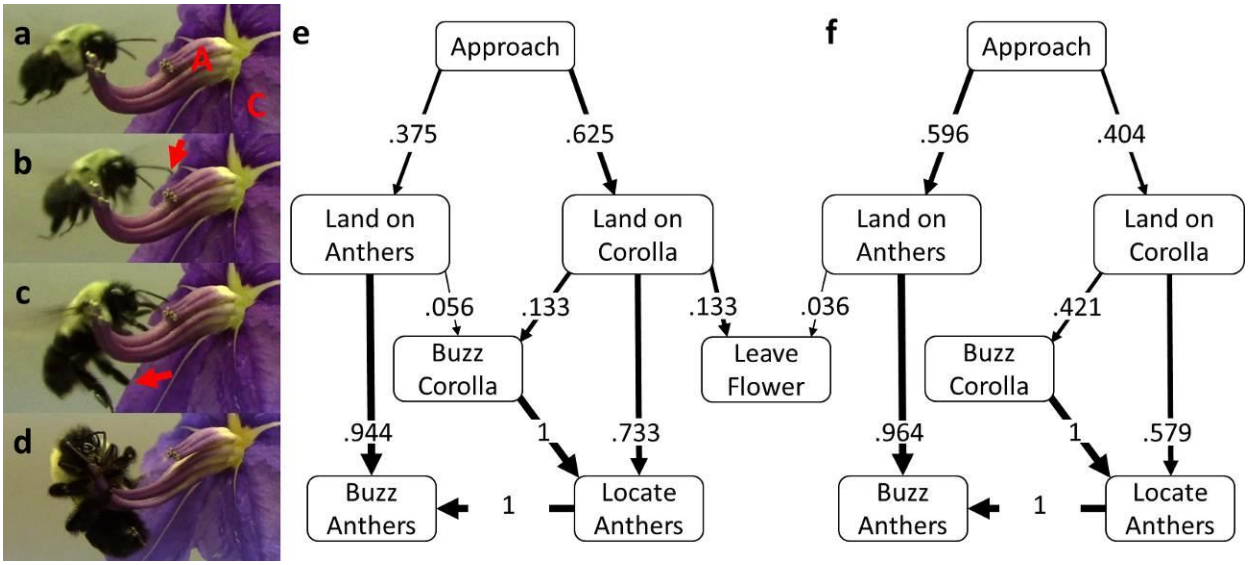


Figure 2. (a-d) Key phases in the first floral visit up until the first buzz or until the bee left the flower: (a) approaching the flower (b) antennal contact with the flower indicated with red arrow (c) landing indicated with red arrow (d) buzzing anthers. Anthers indicated by the red letter "A"; corolla indicated by the red letter "C". (e) Ethogram of the first floral visit. (f) Ethogram of the second floral visit. Arrows indicate the transition from one behavioural component to another. The transition frequency is indicated by both the number and thickness of the arrow. We calculated values by dividing the average number of transitions for a particular component by the total number of transitions derived from a behavioural element. Thus, transition frequencies reflect only the transitions from a given component to any other component (i.e. all transitions from a given component add up to one). We report data from the averaged response of the first and second visit sequence for 48 bees (1 bee discarded for the second sequence, as it made only a single visit).

Figure 3

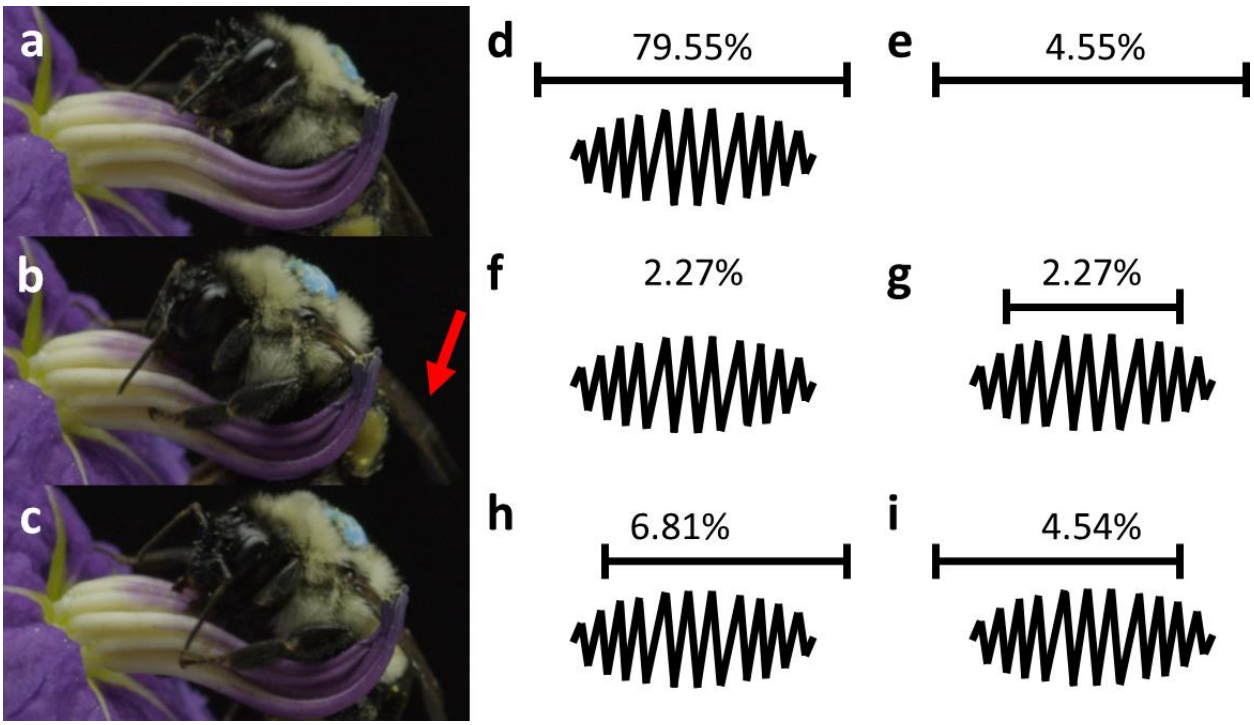


Figure 3. (a-c) Key phases in the floral sonication motor routine: (a) antennal contact and clamping the mandibles on floral tissue, (b) sonicating; blur caused by vibration of wings during buzz indicated with red arrow, (c) releasing mandibles. (d-i) The six kinds of floral sonication motor routines observed in the earliest analysable sequence for each bee on its first floral visit: (d) bite begins before and ends after buzz, (e) a bite with no buzz, (f) a buzz with no bite, (g) buzz begins before and ends after bite, (h-i) buzz and bite are offset positively or negatively. Brackets indicate bite duration, stylized sonograms indicate buzz duration, and percentages indicate the relative frequency of each sequence. We analysed the earliest anther buzz or bite that each bee performed on its first visit, for which the mandibles and antennae were clearly visible (mean number of buzzes/bites to find an analysable sequence ± SE: 3.59 ± 0.52, N = 44 bees).

Figure 4

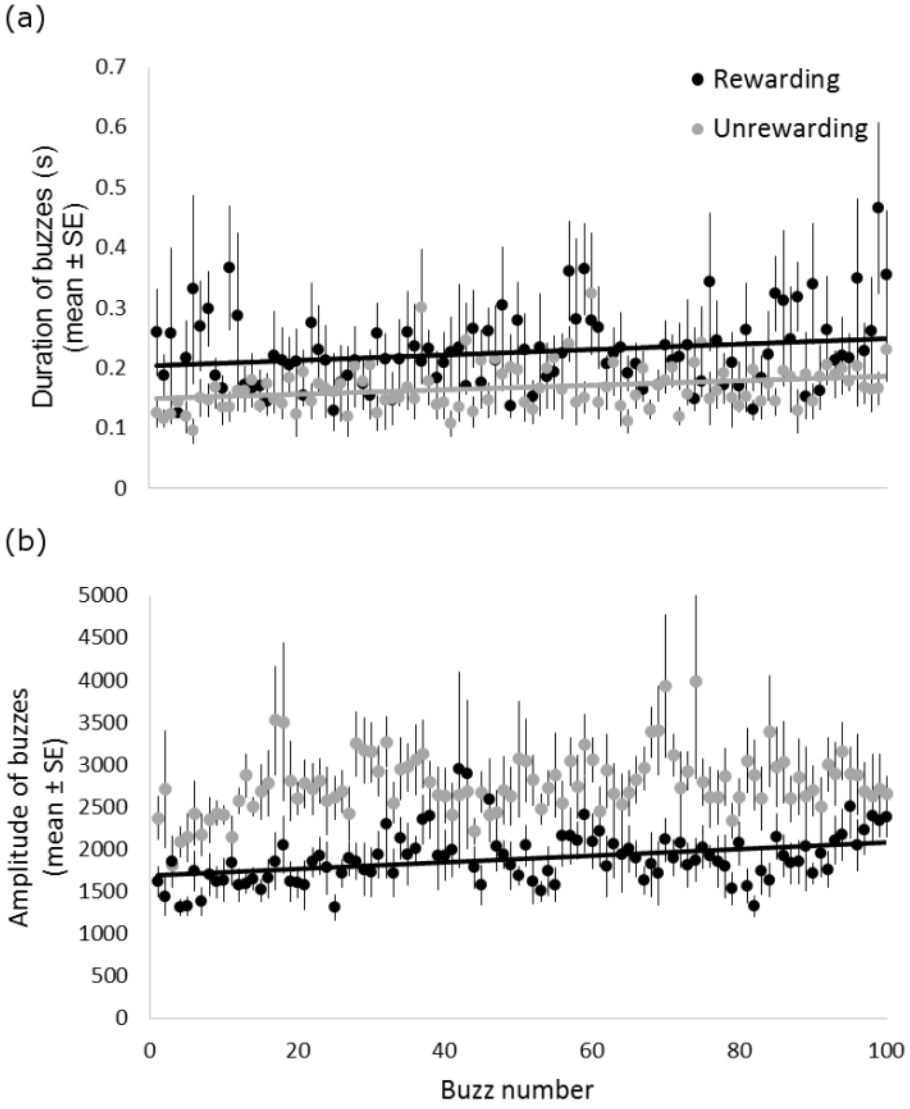


Figure 4. (a) Mean duration (± S.E.) of sonications and (b) mean amplitude (± S.E.) of sonications for the first 100 buzzes. *N* = 6 bees for rewarding and *N* = 7 for unrewarding treatments. Although analyses were performed on log transformed data, the figure shows mean and standard errors for the untransformed data.

Figure 5

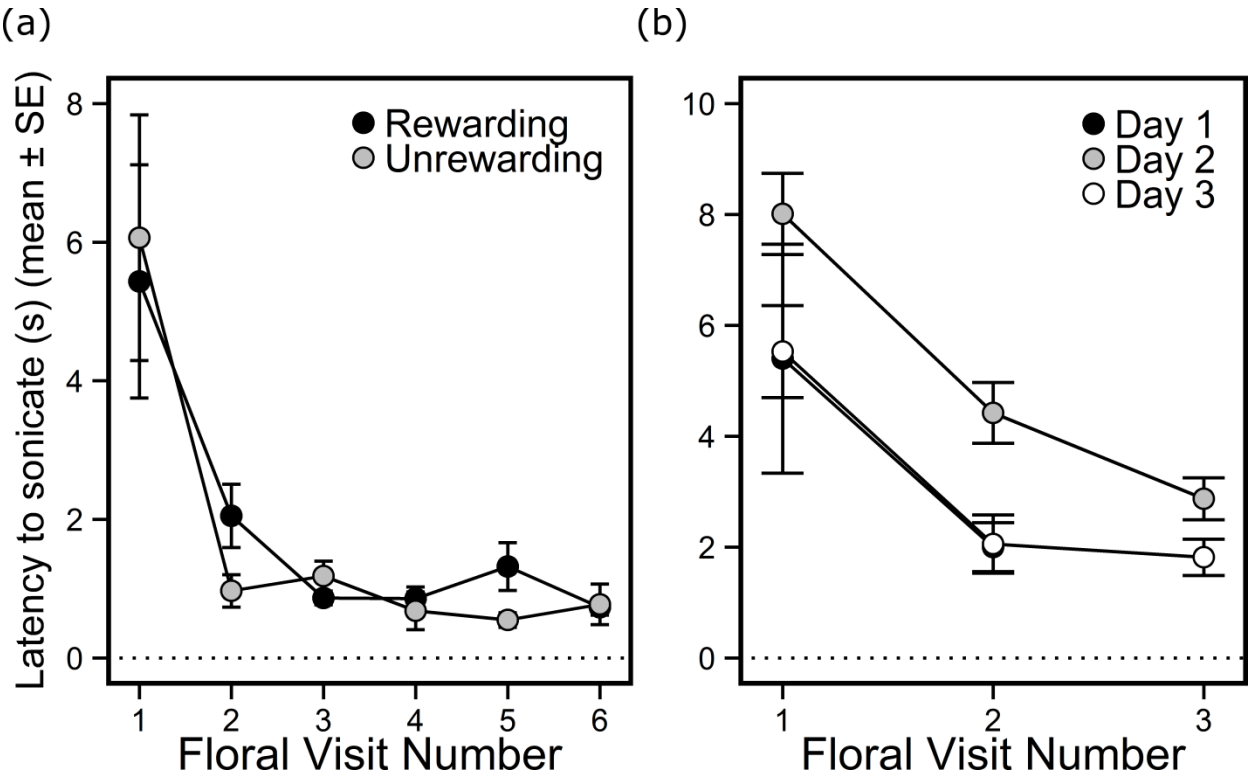


Figure 5. The latency to sonicate (a) on the first six floral visits to a rewarding flower and to an unrewarding flower or (b) on rewarding flowers for the first three floral visits and across days. *N* = 15 and 13 bees for the rewarding and unrewarding treatments, respectively in panel (a). *N* = 10 for bees visiting rewarding flowers in panel (b).

SUPPLEMENTARY MATERIALS

Additional Individual Variation in the Floral Sonication Motor Routine

An analysis of the first approximately 80 analysable buzzes and bites of six bees revealed additional variation in the floral sonication motor routine. Antennal contact with floral tissue always preceded biting of floral tissue. Most bites (94%) resulted in a buzz. Over 98% of sonication events on a bee's first visit involved a single buzz, with the rest resulting in a second buzz. Almost all sonications (97%) were bounded by a single bite (Table S1).

Table S1

Individual variation in the floral sonication motor routine

	Bite				Buzz		
	Preceded by antennal contact	With no buzzes	With one buzz	With two buzzes	With no bites	With one bite	With two bites
Mean percentage	100	5.6	92.8	1.56	0	96.84623	3.153767
SE	0	1.27	1.25	0.72	0	0.840591	0.840591

Data averaged from $N = 6$ bees; mean ± SE buzzes per bee analysed: 83.5 ± 1.43; mean ± SE bites per bee analysed: 78.8 ± 1.8.

The Learning Curve Fitted the Latency Data Well

As an additional check to determine whether Wright's cumulative average model (Martin, n.d.) appropriately described our data, we analysed whether the estimated parameter *a* differed from the actual *a* for each treatment for buzz latency and for anther discovery latency. We found no significant differences (paired *t* tests: rewarding treatment, $N = 15$: buzz latency: $t_{14} = -1.3784$, $P = 0.1897$; anther discovery latency: $t_{15} = -0.3639$, $P = 0.721$; unrewarding treatment, $N = 13$ bees: buzz latency: $t_{12} = 0.2814$, $P = 0.7832$; anther discovery latency: $t_{13} = -0.7096$, $P = 0.4905$).

Body Size Did Not Correlate with Buzz Length or Amplitude

Euthanized bees from the rewarding and unrewarding treatments had their heads removed and photographed at 1.2× using a digital camera with a 5.2 megapixel resolution (DCM500 Microscope CMOS Camera, Microscope Cameras), affixed within the ocular of a stereoscope. We used ImageJ (National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/.) to measure the head width of each bee, as a proxy for body size (head width and body mass or wing length are strongly correlated for *B. impatiens*, $R^2 > 0.95$; Russell, Morrison, Moschonas, Papaj n.d.), using an 84 μm resolution micrometer to calibrate our measurements.

We used a multivariate linear regression model (MLM) to examine how a relationship between the mean length and/or amplitude of the first 100 buzzes was affected by

treatment and/or bee body size. For these models we included the response variables mean buzz length (measured in seconds) and mean buzz amplitude (measured in volume values) and the factors treatment (rewarding flower, unrewarding flower) and body size (measured in micrometres).

Sonication buzz length and amplitude did not correlate significantly with body size or treatment (MLM: buzz length response: $R^2 = 0.05796$, $F_{3,7} = 1.205$, $P = 0.3758$; buzz amplitude response: $R^2 = 0.4295$, $F_{3,7} = 3.51$, $P = 0.0778$).

Body Size Did Not Differ Significantly across Treatments

We used a *t* test to assess whether body size (as measured above) differed across treatments (rewarding versus unrewarding flower; $N = 6$ and 5 bees for the rewarding and unrewarding treatments, respectively) for bees had their buzz amplitude and sonication buzz length measured. Two bees from the unrewarding treatment were discarded because of damage to their heads.

Body size of bees did not differ significantly across treatments (*t* test: $t_9 = 0.614$, $P = 0.55$).

The Pattern of the Latency Drop Was Independent of Flower Reuse

To determine how a bee's scent mark on flowers (Saleh & Chittka, 2006; Wilms & Eltz, 2007) might affect the drop in latency to sonicate, we tested whether the drop also

occurred when a bee switched between fresh flowers. This experiment used 10 bees from two colonies.

We allowed bees a single acceptance to a flower and then replaced that flower with a fresh one for all subsequent acceptances. To accomplish this, we constructed a custom water tube that held two flowers, of which one was concealed under an inverted plastic drinking cup painted the same grey colour as the flight arena. After a bee had made its first acceptance, but before it made its second acceptance, the visited flower was concealed and the unvisited flower was made apparent by rotating it into the position occupied by the visited flower. This change-over took approximately 2 s.

To determine whether there was an effect of experience and treatment on the buzz latency across all six floral visits, we applied a learning curve to each bee's data and analysed the estimated parameters, as described in the main text. We performed a one-way ANOVA to determine whether there were differences in the effect of experience on the learning curve parameters *a* and *b* across treatments (rewarding, unrewarding, replaced). We included the response variable *a* or *b* and the factor treatment. We ran Tukey's post hoc test, using the 'TukeyHSD()' function in R, to determine whether any pairs were significant.

The slope of the learning curve (parameter *b*) did not differ significantly across treatments where the first rewarding flower was replaced or where a single unrewarding or rewarding flower was presented to bees (ANOVA: learning curve's slope (parameter

b), treatment effect: $F_{2,35} = 1.017$, $P = 0.372$; $N = 15$, 13 and 10 bees in rewarding, unrewarding and transfer treatments, respectively; Fig. S1). A Tukey post hoc test likewise revealed no significant differences between treatments for parameter *b*.

However, the learning curve's intercept (parameter *a*) differed significantly across treatments: there was an effect of treatment on the estimated buzz latency for the first floral visit (ANOVA: $F_{2,35} = 5.392$, $P < 0.01$; $N = 15$, 13 and 10 bees in rewarding, unrewarding and transfer treatments, respectively; Fig. S1). A Tukey post hoc test revealed significant differences between the flower replacement treatment and the two single flower treatments (rewarding versus unrewarding: $P = 0.914$; flower replacement versus rewarding: $P < 0.01$; flower replacement versus unrewarding: $P < 0.031$).

In other words, while bees across treatments similarly reduced their buzz latency with experience, the buzz latency initially differed between groups (before flowers had been revisited by bees or replaced by the experimenter). This result was reflected in a separate analysis in which we analysed the buzz latency for only the first and second floral visits. We found that differences in the buzz latency across treatments disappeared by the second floral visit, demonstrating that the initial differences in buzz latency and the buzz latency on subsequent floral visits were independent of flower reuse (ANOVAs: first visit: treatment effect: $F_{2,43} = 6.143$, $P < 0.0037$; second visit: treatment effect: $F_{2,40} = 1.112$, $P = 0.339$; $N = 18(17)$, 18(16), and 10(10) bees in rewarding, unrewarding and replacement treatments, respectively, with parenthetical numbers indicating *N* on the second visit). Furthermore, the initially high buzz latency in the replacement treatments

was likely a result of the unusually high frequency of nectar probing before sonication in this treatment (50% of bees in the replacement treatment, versus 0% of bees in the rewarding and unrewarding treatments). In fact, in the replacement treatment, the mean buzz latency of nectar probing bees was 30.9 s, compared to 8.6 s for bees that did not nectar-probe before sonicating.

Figure S1

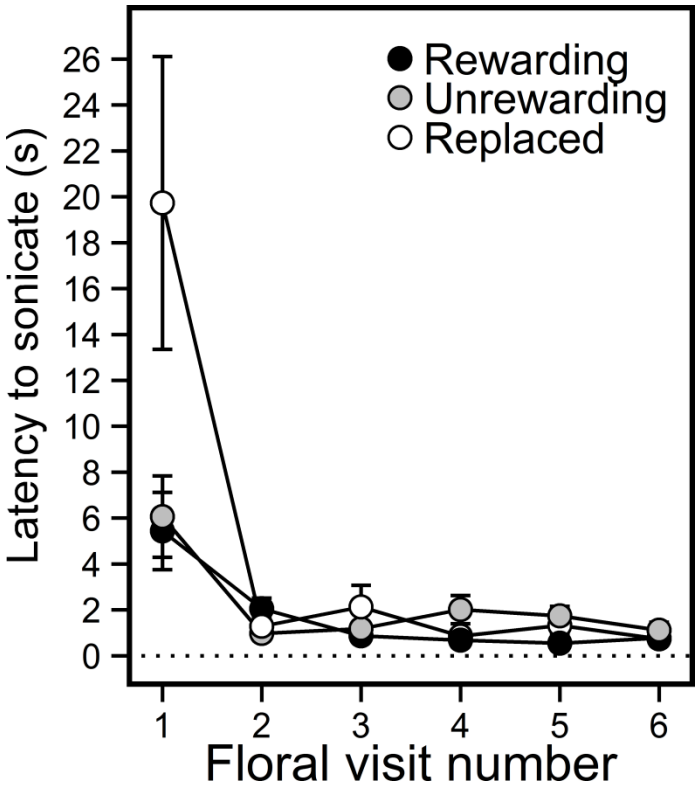


Figure S1. Mean ± SE latency to sonicate on the first six floral visits to a rewarding flower (*N* = 15 bees), an unrewarding flower (*N* = 13 bees) and a replaced rewarding flower (*N* = 10 bees).

Table S2

Number of buzzes analysed and delivered to each floral structure

Treatment/bee	No. of buzzes	No. of anther buzzes	No. of corolla buzzes*
Rewarding			
1	291	289	2
2	542	542	0
3	176	167	9
4	498	497	1
5	304	277	27
6	214	213	1
Mean	337.5	330.8333	6.666667
SE	66.99239	68.57896	4.687572
Unrewarding			
1	437	386	51
2	385	385	0
3	391	384	7
4	517	472	45
5	431	336	95
Mean	432.2	392.6	39.6
SE	23.60169	22.00364	17.11607

SUPPLEMENTARY REFERENCES

Hothorn, T., Bretz F., Westfall, P., Heiberger, R.M., Scheutzenmeister, A., & Scheibe, S. (2015). *Simultaneous inference in general parametric models* (R package version 1.4-1). Vienna, Austria: R Foundation for Statistical Computing. http://CRAN.R-project.org/package=multcomp.

Martin, J. R. (n.d.). *What is a learning curve? Management and accounting web*. http://maaw.info/LearningCurveSummary.htm (accessed May 2016).

Russell, A.L., Morrison, S.J., Moschonas, E.H., Papaj, D.R. (n.d.). *Patterns of pollen and nectar foraging specialization by bumblebees over multiple timescales*. Manuscript in preparation.

Saleh, N., & Chittka, L. (2006). The importance of experience in the interpretation of conspecific chemical signals. *Behavioral Ecology and Sociobiology, 61*, 215–220.

Wilms, J., & Eltz, T. (2007). Foraging scent marks of bumblebees: Footprint cues rather than pheromone signals. *Naturwissenschaften, 95*, 149–153.

APPENDIX E

HOW A GENERALIST BEE ACHIEVES HIGH EFFICIENCY OF POLLEN COLLECTION ON DIVERSE FLORAL RESOURCES

FULL TITLE: How a generalist bee achieves high efficiency of pollen collection on diverse floral resources

Avery L. Russell[a,b*], Stephen L. Buchmann[b], and Daniel R. Papaj[b]

[a] Graduate Interdisciplinary Program in Entomology and Insect Science, University of Arizona, Tucson, AZ, 85721. USA

[b] Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, 85721. USA.

*Corresponding author

This manuscript has been submitted to *Current Biology*.

SUMMARY

A fundamental question in biology is how generalist animals use diverse resources efficiently [1-3]. This question has been extensively addressed for generalist pollinators foraging on diverse plant species that vary greatly in floral morphology. For instance, generalist bees use instrumental (associative) learning to acquire routines specific to each flower type to extract nectar efficiently [4-6]. Such associative learning allows a generalist bee to use novel species that it may never have encountered in its evolutionary history, and may have facilitated the diversification of floral form [7-8]. Yet, nectar is not the only floral reward, and instrumental learning may not be the only means by which flexible behavior is achieved. While bees collect pollen from diverse species [9-11], few studies have examined if and how high efficiency of collection is attained. We show that generalist bumble bees exhibit flexible and effective pollen collection by switching between two distinct routines: “scrabbling” when pollen is abundant, and “sonicating” when pollen is scarce. Switching between these behaviors is regulated by the interplay between two ubiquitous floral cues: chemical anther cues stimulating sonication, and mechanical pollen cues suppressing sonication (and eliciting scrabbling). This mechanism of behavioral flexibility likely allows generalist bees to handle diverse anther morphologies efficiently and may have facilitated the recurrent evolution of plant species that conceal pollen rewards via pored floral morphology. Whereas effective nectar foraging requires associative learning of unique routines for each flower type, we found no indication that the pollen collection flexibility described in this study requires it.

KEY WORDS: *bumble bee, floral sonication, pollen foraging, pollinator mutualism, floral evolution, specialization, behavioral flexibility, learning*

RESULTS

Generalist species of bees foraging for floral rewards are one of our most thoroughly studied examples of foraging ecology. Bees must collect nectar and pollen, their primary source of carbohydrates and pollen, respectively. However, nearly all literature on flexible foraging behavior by bees concerns the collection of nectar. While it is reasonable to conjecture that bees adjust their behavior to collect pollen effectively from diverse plant species, the nature of this flexibility and its implications for floral evolution have scarcely been examined in comparison to nectar collection.

One place to look for flexibility is in the two means by which pollen is collected from biotically pollinated flowers of varying morphology. Pollen varies from being entirely exposed to being entirely concealed (Figure 1). The pollen of most angiosperm flowers is exposed, to varying degrees, on the surface of the anthers (Figure 1g-i). Bees use their legs and mandibles to knock exposed pollen free via a behavior called scrabbling [12] (see [13] for a video). Six-8% of plant species (>22,000 species across >72 angiosperm families; [14-15]) entirely conceal pollen within specialized tube-like anthers or, less commonly, corollas (poricidal floral morphology; [15-17]; Figure 1a-c). To expel concealed pollen, bees rapidly contract their indirect flight muscles while biting the

anthers or corolla, via a behavior called floral sonication (or buzzing) [18-19]. Scrabbling is never observed on poricidal species, and is inefficient for removing and transferring pollen from flowers that partially conceal pollen (e.g., [20-22]; Figure 1d-f). Can bees adjust their behavior to collect pollen effectively from these types of flowers?

While pollination via the buzz mechanism is generally considered a relatively specialized interaction [14-15], we and others have sometimes observed bees sonicating on non-poricidal flowers (Table S1). Sonication on non-poricidal species is not consistently expressed: not only might one bee scrabble while another sonicates, but individual bees have been observed sonicating some flowers and scrabbling on others of the same species (e.g., [14,23-24]). These observations lead us to propose that generalist bees may be able to adjust when they use sonication versus scrabbling in order to effectively collect pollen from plant species with flowers of diverse morphologies.

We devised laboratory experiments to examine the floral cues that regulate the flexibility of pollen foraging behavior in the generalist bumble bee (*Bombus impatiens*) on non-poricidal species. We first tested how the amount of exposed pollen on the anthers affects expression of sonication (Figure 2). We then characterized two sets of cues, anther-based chemical cues and pollen-based mechanical cues (Figure 3,4), which together account for expression of, and switching between, sonication and scrabbling. Finally, we assessed whether the conditional expression of floral sonication on non-poricidal species resulted in effective pollen collection, thereby providing a mechanism by which bees might

effectively collect pollen from plant species that partially conceal their pollen (e.g., Figure. 1d-f).

Bumble bees sonicate the anthers of non-poricidal flowers but pollen suppresses this response

Bees flexibly switched between sonication and scrabbling between floral visits when foraging from non-poricidal flowers (Figure S3). Bees rarely sonicated previously unvisited flowers of *Begonia descoleana*, a non-poricidal species that offers only pollen as a reward, but readily sonicated flowers depleted of pollen during previous visits (Figure 2a,b; paired Wilcoxon: $V = 36$, $P < 0.0143$).

This result is probably an effect of a difference in pollen availability and not of correlated factors, such as a mark left by the bee on flowers: bees readily sonicated flowers of *Begonia hybrida*, a non-poricidal, pollenless hybrid (Figure 2c), and sonicated them on a higher proportion of visits relative to visits to *B. hybrida* flowers whose anthers had been supplemented with pollen (Figure 2d,e; *t*-test: $t_{19.087} = -7.81$, $P < 0.0001$). Even naïve bees on their initial visit only sonicated pollenless flowers and only scrabbled pollen-supplemented flowers (Figure S4a). Bees sonicated pollen-supplemented flowers more frequently after they had been depleted of added pollen during previous visits (Figure 2f; paired Wilcoxon: $V = 45$, $P < 0.0092$). Additionally, bees sonicated flowers more frequently that were initially enriched with smaller amounts of pollen (Figure 2g; ANOVA: $F_{2,19} = 28.18$, $P < 0.0001$). In all cases, bees landing on pollen-supplemented flowers collected that pollen (Figure S1b). Bees never scrabbled on bare flowers.

Chemical extracts of anthers from non-poricidal or poricidal species elicit sonication by bees

Bees sonicated on a greater proportion of visits to surrogate flowers treated with a pentane extract of live anthers versus on visits to surrogate flowers treated with a pentane control (Figure 3). Surrogate flowers consisted of live corollas bearing artificial foam anthers treated with the pentane extract or control. In both treatments (surrogates and extract either made from non-poricidal, rewardless *B. hybrida*, Figure 3a, or from poricidal *Solanum houstonii*, Figure 3b), visits to anther extract-treated surrogates resulted in sonication significantly more often than visits to surrogates treated with a pentane control (Wilcoxon: Figure 3c; *B. hybrida*: $V = 60$, $P < 0.019$; Figure 3d; *S. houstonii*: $V = 5.44$, $P < 0.0039$). All bees but one made their first buzz on an anther extract-treated surrogate flower. Bees never sonicated corollas and never scrabbled on surrogate flowers.

Pollen-like mechanosensory stimuli suppress sonication

Patterns of behavior by bees collecting plastic microspheres and cellulose powder (both 20µ diameter particles) similar in size to pollen were similar to patterns of bee behavior observed in experiment 1 (Figure 4). Bees were more prone to sonicate *B. hybrida* flowers depleted of plastic or cellulose in previous visits (Figure 4b; for both treatments, paired Wilcoxon: $V = 28$, $P < 0.016$), and were more prone to sonicate flowers

supplemented with less cellulose (Figure 4c; ANOVA: $F_{2,19} = 40.08$, $P < 0.0001$). Bees visiting flowers supplemented with 20µ plastic or cellulose particles always collected the particles (Figure 4a,S1c). We used cellulose because it elicited equivalent patterns of behavior as plastic (Figure4,S4d) and much larger cellulose particles stick to the anthers (unlike larger plastic microspheres).

Cellulose particles (180µ diameter) much larger than pollen typically collected by generalist bees [25] did not suppress sonication and bees did not scrabble for these particles, as they did for the 20µ cellulose powder, plastic microspheres or pollen. Specifically, the proportion of landings involving sonication by bees visiting *B. hybrida* flowers that presented large cellulose particles on their anthers was not significantly different from that by bees alighting on unmanipulated bare flowers (Figure 4f; Wilcoxon: $W = 30$, $P = 0.873$).

Bees removed pollen at a higher rate by sonicating than by scrabbling when non-poricidal flowers presented small amounts of pollen

Because bees switched from scrabbling to sonicating when pollen was depleted on live flowers, we investigated the benefit of using one versus the other routine when pollen was depleted. Bees that only sonicated pollen-supplemented artificial (all foam) non-poricidal flowers (Figure S1f) collected pollen at a significantly higher rate (52% higher) than bees that only scrabbled for pollen (*t*-test: $t_{17.123} = 2.4693$, $P < 0.025$; mean mg pollen/s ± SE: scrabbled, 0.024 ± 0.004; sonicated, 0.036 ± 0.003; $N = 11$ bees per

treatment). Neither length of time foraging nor amount of pollen collected alone accounted for the significant difference in the higher collection rate by sonication over scrabbling (Wilcoxon: time foraging, $W = 42$, $P = 0.243$, mean seconds ± SE: sonicated, 58.79 ± 5.36; scrabbled, 78.50 ± 12.55; amount collected, $W = 87$, $P = 0.0869$; mean mg ± SE: sonicated, 2.15 ± 0.30; scrabbled, 1.47 ± 0.15; $N = 11$ bees per treatment).

DISCUSSION

The consequences of flexibility in generalist pollinator behavior for the evolution of plant-pollinator mutualisms have been considered extensively in the context of nectar foraging. Flexible nectar foraging behavior via learning can facilitate floral trait evolution (e.g., [26-29]) and species invasions [8], for example, by allowing pollinators to use floral morphologies they have not previously encountered (e.g., [8,30]). Our findings suggest that generalist bees can also flexibly adjust pollen-collecting behavior to make effective use of diverse floral resources. These pollinators may thus exert similar impacts on floral diversity in the context of pollen collection, particularly with regard to the form and arrangement of anthers and associated structures.

Our results have particularly important implications for the widespread and repeated evolution of poricidal floral morphology from non-poricidal ancestors [14-15,31], particularly if poricidal morphology evolves gradually, via a series of intermediate stages, as commonly presumed for traits [28]. Under a scenario of gradual evolution,

intermediate stages will have pollen partially concealed to varying degrees (e.g., Figure 1d-f). Scrabbling alone is ineffective at removing unexposed pollen from such flowers [20-22] and thus such intermediates are at a potential disadvantage. However, our findings suggest that a combination of scrabbling and sonication might result in effective pollen collection, and thus effective pollen transfer, for plants with anthers of these intermediate types. As pollen is progressively concealed during this evolutionary process, sonication would be expressed more and more during pollen collection, until eventually, when flowers are fully poricidal and pollen entirely concealed, it would be the only behavior used to extract pollen. To test this proposed scenario, future work should evaluate whether bees are indeed able to collect and transfer pollen relatively efficiently from plant species that partially conceal their pollen, using a combination of scrabbling and sonication.

While flexibility in behavior is key to the evolutionary scenario above, our results suggest that flexibility in pollen collection involves a different mechanism than that involved in nectar collection. Whereas bees use instrumental learning to acquire and refine unique routines specific to particular flower types to extract nectar (e.g., [4-6]), bumble bees exhibit flexibility by switching between two stereotyped pollen collection routines, floral sonication and scrabbling. Switching is regulated by the interacting effects of two floral cues: chemosensory anther cues and mechanosensory pollen cues. Importantly, chemosensory anther cues elicited sonication and mechanosensory pollen cues suppressed sonication (and elicited scrabbling) by even naïve bees on their first floral visit (Figure S4a-c), suggesting that use of either routine is innately specified.

Although switching did not appear to require instrumental learning in our study, the effectiveness of collection routines might nevertheless be altered with experience. To improve pollen collection efficiency, bees may learn when to switch between scrabbling and sonicating, or adjust subtle characteristics of the two routines themselves (e.g., [32,19]). Sonication has a strong innate component and is affected little by experience [19]; however, scant work has examined whether bees learn to scrabble (see [33]). Furthermore, experience could influence how bees collect pollen in response to naturally varying floral features other than pollen presentation. Across taxa, pollen varies greatly in stickiness, clumping, size, and surface structure [25,34-36]. Bees might learn to adjust scrabbling, sonication, or how these behaviors are combined, so as to maximize pollen collection.

At present, we are relatively ignorant of the relative costs and benefits of scrabbling and sonication. While we have shown that sonication results in higher rates of collection of exposed pollen at low levels (when bees would switch from using scrabbling to using sonication on live flowers), we have not assessed what costs might be involved in achieving these higher rates. For example, sonication is likely energetically expensive relative to scrabbling: flight (which, like sonication, uses indirect flight muscles) is on average 50% more energetically expensive than walking locomotion (which, like scrabbling, involves leg motion) [37]. It would be useful to evaluate the extent to which energetic and other costs influence the relative use of sonication and scrabbling, especially on different anther morphologies.

EXPERIMENTAL PROCEDURES

Fully described in Supplemental Experimental Procedures.

AUTHOR CONTRIBUTIONS

Conceptualization and writing, A.L.R. and D.R.P.; Investigation, A.L.R. and S.L.B.; Analysis, A.L.R.; All authors revised the manuscript.

ACKNOWLEDGEMENTS

We thank Judith Bronstein and Shayla Salzman for comments, Pollen Collection and Sales (Lemon Cove, CA) for pollen, the FMC Corporation for Avicel PH-200 NF, Andrew Walzer at Thermo Scientific for microspheres, Abreeza Zegeer for greenhouse care, China Rae Newman and Eleni Moschonas for assistance running trials, and Wulfila Gronenberg for microscopes and technical support. This work was supported by the Graduate & Professional Student Council of the University of Arizona and the National Science Foundation (IOS-1257762).

REFERENCES

1. Loeuille, N. 2010. Consequences of adaptive foraging in diverse communities. *Functional* Ecology *24*, 18-27.

2. Wright, T.F., Eberhard, J.R., Hobson, E.A., Avery, M.L., and Russello, M.A. 2010. Behavioral flexibility and species invasions: the adaptive flexibility hypothesis. Ethol. Ecol. Evol. *22*, 393-404.

3. Baudrot, V., Perasso, A., Fritsch, C., Giraudoux, P., and Raoul, F. 2016. The adaptation of generalist predators' diet in a multi-prey context: insights from new functional responses. Ecology. *97*, 1832-1841.

4. Lewis, A.C. 1993. Learning and the evolution of resources: pollinators and flower morphology. In Insect learning: ecological and evolutionary perspectives, Papaj, D.R., Lewis, A.C., eds. (New York: Chapman & Hall). pp. 219–242.

5. Laverty, T.M. 1994. Bumble bee learning and flower morphology. Anim. Behav. *47*, 531-545.

6. Gegear, R.J., and Laverty, T.M. 1995. Effect of flower complexity on relearning flower-handling skills in bumble bees. Can. J. Zool. *73*, 2052-2058.

7. Schiestl, F.P., and Johnson SD. 2013. Pollinator-mediated evolution of floral signals. Trends Ecol. Evolut. *28*, 307–315.

8. Bartomeus, I., Fründ, J., & Williams, N.M. 2016. Invasive plants as novel food resources, the pollinators' perspective. In Biological Invasions and Behavior, Sol, D. and Weis, J., eds. (Cambridge: Cambridge University Press). pp. 119-132.

9. Simpson, B.B., and Neff, J.L. 1981. Floral rewards: alternatives to pollen and nectar. Ann. Missouri Bot. Gard. *68*, 301–322.

10. Kevan, P.G., and Baker, H.G. 1983. Insects as flower visitors and pollinators. *Ann Rev Entomol. 28*, 407-453.

11. Nicolson, S.W. 2011. Bee food: the chemistry and nutritional value of nectar, pollen and mixtures of the two. Afr. Zool. *46*, 197-204.

12. Thorp, R.W. 2000. The collection of pollen by bees. Plant. Syst. Evol. *222*, 211-223.

13. Russell, A.L., and Papaj, D.R. 2016. Artificial pollen dispensing flowers and feeders for bee behaviour experiments. J. Pollinat. Ecol. *18*, 13-22.

14. Buchmann, S.L. 1983. Buzz pollination in angiosperms. In Handbook of experimental pollination biology, Jones, C.E., Little, R.J., eds. (New York: Van Nostrand Reinhold). pp. 73–113.

15. De Luca, P.A., and Vallejo-Marín, M. 2013. What's the 'buzz' about? The ecology and evolutionary significance of buzz-pollination. Curr. Opin. Plant Biol. *16*, 429-435.

16. Houston, T.F., and Ladd, P.G. 2002. Buzz pollination in the Epacridaceae. Aust. J. Bot. *50*, 83-91.

17. Corbet, S.A., and Huang, S.Q. 2014. Buzz pollination in eight bumblebee-pollinated *Pedicularis* species: does it involve vibration-induced triboelectric charging of pollen grains? Ann. Bot. *114*, 1665-1674.

18. Buchmann, S.L., and Cane, J.H. 1989. Bees assess pollen returns while sonicating *Solanum* flowers. Oecologia. *81*, 289-294.

19. Russell, A.L., Leonard, A.S., Gillette, H.D., and Papaj, D.R. 2016. Concealed floral rewards and the role of experience in floral sonication by bees. Anim. Behav. *120*, 83-91.

20. King, M.J., and Ferguson, A.M., 1994. Vibratory collection of *Actinidia deliciosa* (Kiwifruit) pollen. Ann. Bot. *74*, 479-482.

21. Javorek, S.K., Mackenzie, K.E., and Vander Kloet, S.P. 2002. Comparative pollination effectiveness among bees (Hymenoptera: Apoidea) on lowbush blueberry (Ericaceae: *Vaccinium angustifolium*). Ann. Entomol. Soc. Am. *95*, 345-351.

22. Pomeroy, N., and Fisher, R.M. 2002. Pollination of kiwifruit (*Actinidia deliciosa*) by bumble bees (*Bombus terrestris*): effects of bee density and patterns of flower visitation. New Zeal. Entomol. *25*, 41-49.

23. Buchmann, S.L. 1985. Bees use vibration to aid pollen collection from non-poricidal flowers. J. Kans. Entomol. Soc. *58*, 517-525.

24. Pellmyr, O. 1985. Pollination ecology of *Cimicifuga arizonica* (Ranunculaceae). Bot. Gaz. *146*, 404-412.

25. Roberts, R.B., and Vallespir, S.R. 1978. Specialization of hairs bearing pollen and oil on the legs of bees (Apoidea: Hymenoptera). Ann. Entomol. Soc. Am. *71*, 619-626.

26. Sargent, R.D. 2004. Floral symmetry affects speciation rates in angiosperms. Proc. Nat. Acad. Sci. *271*, 603-608.

27. Gómez, J.M., Perfectti, F., and Lorite, J. 2015. The role of pollinators in floral diversification in a clade of generalist flowers. Evolution *69*, 863-878.

28. Guzmán, B., Gómez, J.M., and Vargas, P. 2015. Bees and evolution of occluded corollas in snapdragons and relatives (Antirrhineae). Perspect. Plant Ecol. Evol. Syst. *17*, 467-475.

29. Rojas-Nossa, S.V., Sánchez, J.M., and Navarro, L. 2016. Nectar robbing: a common phenomenon mainly determined by accessibility constraints, nectar volume and density of energy rewards. Oikos. *125*, 1044-1055.

30. Chittka, L., and Thomson, J.D. 1997. Sensori-motor learning and its relevance for task specialization in bumble bees. Behav. Ecol. Sociobiol. *41*, 385-398.

31. Vallejo-Marín, M., Da Silva, E.M., Sargent, R.D., and Barrett, S.C.H. 2010. Trait correlates and functional significance of heteranthery in flowering plants. New. Phyt. *188*, 418-425.

32. Morgan, T., Whitehorn, P., Lye, G. C., and Vallejo-Marin, M. (2016). Floral sonication is an innate behaviour in bumblebees that can be fine-tuned with experience in manipulating flowers. J. Insect. Behav. *29*, 233-241.

33. Raine, N.E., and Chittka, L. 2007. Pollen foraging: learning a complex motor skill by bumblebees (*Bombus terrestris*). Naturwissenschaften *94*, 459-464.

34. Hesse, M. 1981. Pollenkitt and viscin threads: their role in cementing pollen grains. Grana. *20*, 145-152.

35. Pacini, E., and Hesse, M. 2005. Pollenkitt - its composition, forms and functions. Flora *5*, 399-415.

36. Lunau, K., Piorek, V., Krohn, O., and Pacini, E. 2015. Just spines—mechanical defense of malvaceous pollen against collection by corbiculate bees. Apidologie *46*, 144-149.

37. Balfour, N.J., Gandy, S., and Ratnieks, F.L.W. 2015. Exploitative competition alters bee foraging and flower choice. Behav. Ecol. Sociobiol. *69*,1731-1738.

FIGURE LEGENDS

Figure 1. Examples of poricidal and non-poricidal flowers. Three species conceal pollen either within poricidal anthers: (a) *Senna covesii* and (b) *Solanum elaeagnifolium*; or a poricidal corolla: (c) *Pedicularis groenlandica*. Three species that partially conceal pollen, to varying degrees: (d) *Dianella revoluta* (e) *Dodonaea microzyga* (f) *Asterolasia grandiflora*. Three non-poricidal species display pollen openly on anthers: (g) *Deppea splendens* (h) *Aloe cryptopoda* (i) *Phacelia campanularia*. Photographs: (a,b, g-i): Avery Russell; (c): Walter Siegmund, licensed by CC BY-SA 3.0; (d-f): Kevin Thiele, licensed by CC BY 2.0. See also Table S1.

Figure 2. Mean percentage landings (± SE) by bees resulting in sonication in treatments where the availability of pollen presented by *Begonia descoleana and B. hybrida* was varied. (a) *B. descoleana* and (c) *B. hybrida* in its natural state and (d) *B. hybrida* supplemented with 2mg pollen. Mean percentage landings resulting in sonication (b) of *B. descoleana* flowers on the first landings versus repeat landings; (e) of *B. hybrida* pollenless flowers or pollen-supplemented flowers; (f) of pollen-supplemented flowers on the first landings to each flower in an array versus repeat landings and; (g) of flowers initially supplemented with 1, 2, or 4mg of pollen. (b) $N = 9$ bees for *B. descoleana* treatment. (e,f) $N = 12$, 11 bees for the *B. hybrida* pollenless and pollen-supplemented treatment, respectively. (g) $N = 7$, 7, and 8 bees for the 1, 2 and 4mg pollen treatments,

respectively. Different letters above bars indicate significant differences at $p<0.05$ according to a Wilcoxon test, *t*-test, or Tukey's post hoc test. See also Figure S1-S4.

Figure 3. Mean percentage landings (± SE) resulting in sonication by bees on anther extract surrogates and on pure pentane treated surrogates made using non-poricidal *Begonia hybrida* or poricidal *Solanum houstonii*. (a) *B. hybrida* surrogates. (b) *S. houstonii* surrogates. (c, d) Mean percentage landings (± SE) on surrogates treated with anther extract or pure pentane resulting in sonication. $N = 11$ bees per treatment. Different letters above bars indicate significant differences at $p<0.05$ according to a Wilcoxon test.

Figure 4. Mean percentage landings (± SE) by bees resulting in sonication in treatments where the availability or size of plastic microspheres or cellulose particles presented by *Begonia hybrida* was varied. (a) Representative forager that collected plastic microspheres (black arrow). (c) *B. hybrida* with 4mg of 20µ size cellulose particles. Mean percentage landings (± SE) resulting in sonication (b) on flowers supplemented with 2mg 20µ plastic microspheres on first landings to each flower in an array versus repeat landings; (d) on flowers supplemented with 2mg 20µ cellulose particles on first landings to each flower in an array versus repeat landings; (e) on flowers initially supplemented with 1, 2, or 4mg of 20µ cellulose particles, and; (f) on bare flowers versus flowers enriched with 180µ cellulose particles. (b) $N = 7$ bees per treatment. (d,e) $N = 7$ bees each for the 1, 2 and 4mg cellulose treatments. (f) $N = 8$ per treatment. Different

letters above bars indicate significant differences at $p<0.05$ according to a Wilcoxon test or Tukey's post hoc test. See also Figure S1,S2,S4.

FIGURES

Figure 1

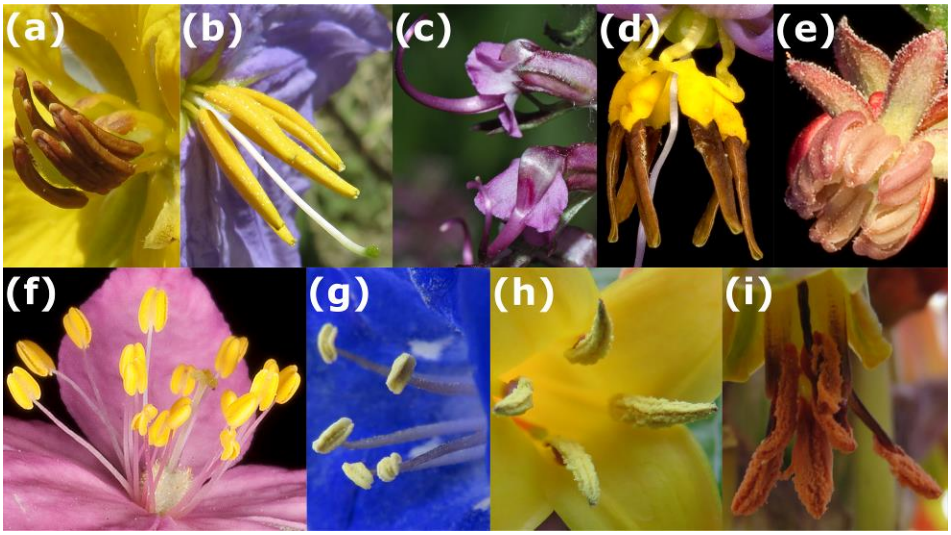


Figure 2

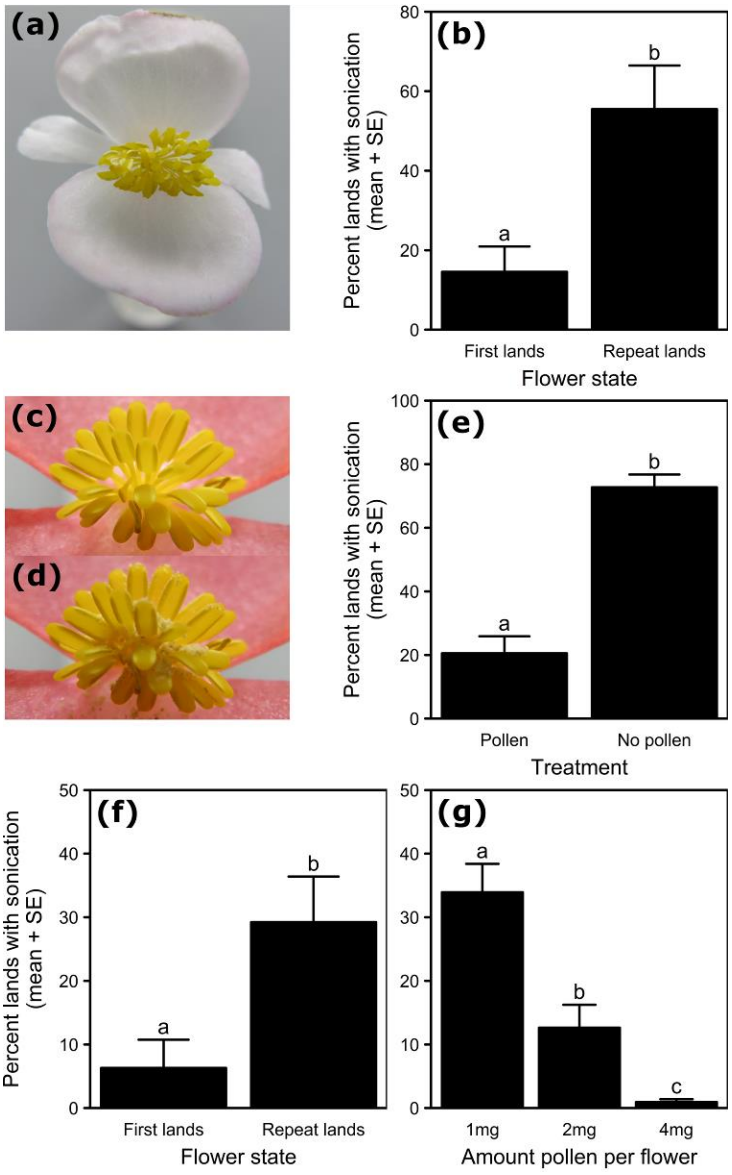

(a)
(b)
Percent lands with sonication (mean + SE)
80
60
40
20
0
a
b
First lands
Repeat lands
Flower state
(c)
(d)
(e)
100
80
60
40
20
0
a
b
Pollen
No pollen
Treatment
(f)
50
40
30
20
10
0
a
b
First lands
Repeat lands
Flower state
(g)
a
b
c
1mg
2mg
4mg
Amount pollen per flower

Figure 3

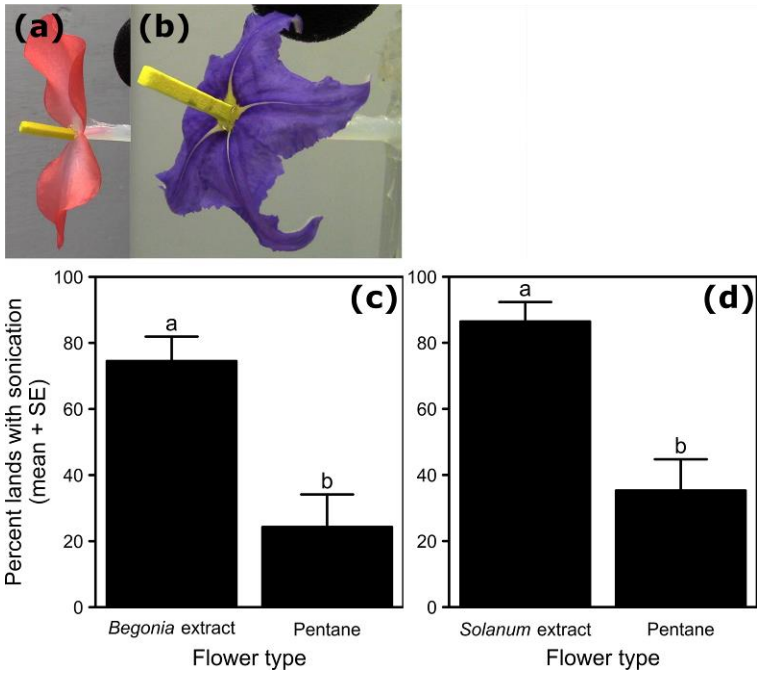
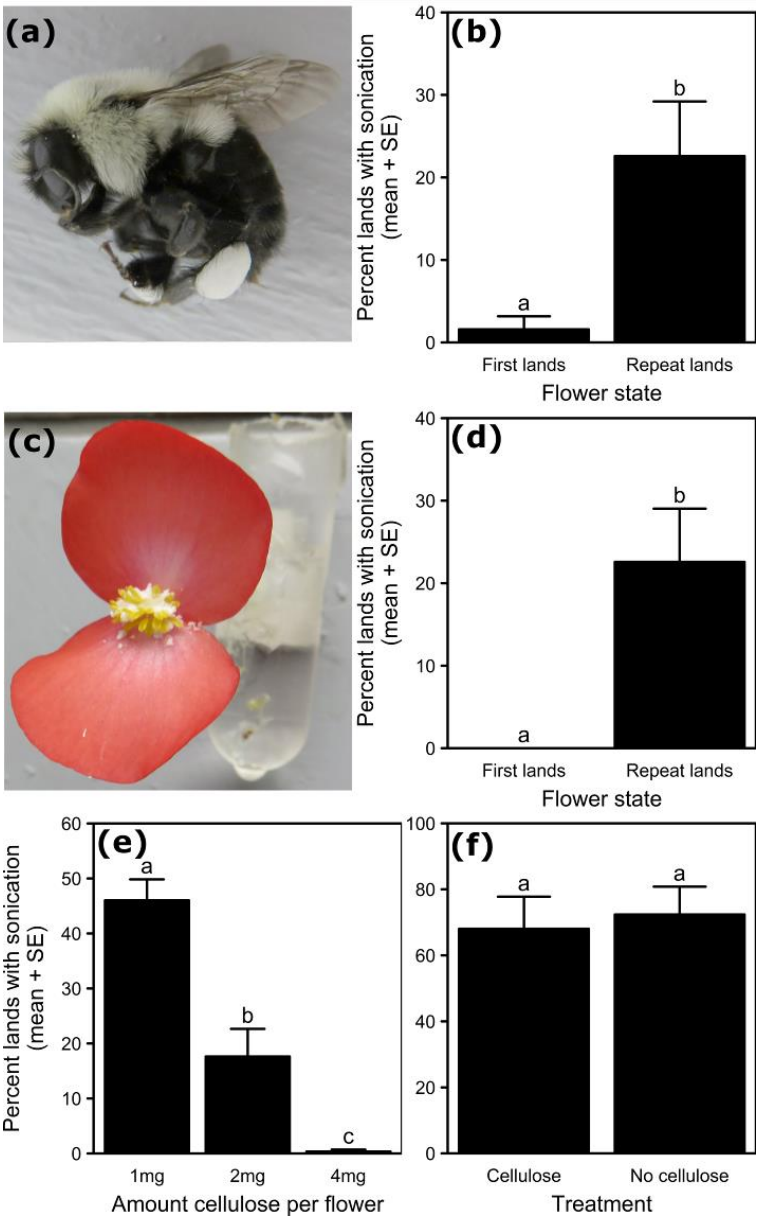

(a)
(b)
(c)
(d)
Percent lands with sonication (mean + SE)
100
80
60
40
20
0
a
b
Begonia extract
Pentane
Flower type
Solanum extract
Pentane
Flower type

Figure 4


(a)
(b)
Percent lands with sonication (mean + SE)
40
30
20
10
0
a
b
First lands
Repeat lands
Flower state
(c)
(d)
Percent lands with sonication (mean + SE)
40
30
20
10
0
a
b
First lands
Repeat lands
Flower state
(e)
Percent lands with sonication (mean + SE)
60
50
40
30
20
10
0
a
b
c
1mg
2mg
4mg
Amount cellulose per flower
(f)
100
80
60
40
20
0
a
a
Cellulose
No cellulose
Treatment

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL DATA

Table S1, related to Figure 1: Non-poricidal species sonicated by bees

Family	Genus	Anthers directly accessible?	Sonicating bee(s)	Bees sonicated stamens?	Reference
Asteraceae	*Senecio vulgaris*	Yes	*Bombus edwardsii*	Yes	[14,S1]
Begoniaceae	*Begonia cucullata*	Yes	*Bombus impatiens*	Yes	A. Russell pers. obs.
Begoniaceae	*Begonia descoleana*	Yes	*Bombus impatiens*	Yes	This manuscript
Begoniaceae	*Begonia 'Angel Wing'*	Yes	*Bombus impatiens*	Yes	A. Russell pers. obs.
Begoniaceae	*Begonia 'Dragon Wing'*	Yes	*Bombus impatiens*	Yes	This manuscript
Begoniaceae	*Begonia odorata*	Yes	*Bombus impatiens*	Yes	A. Russell pers. obs.
Bignoniaceae	*Tecoma alata*	Yes	*Bombus impatiens*	Typically	A. Russell pers. obs.
Bignoniaceae	*Tecoma stans*	Yes	*Bombus sonorus*	Yes	D. Papaj and K. Mauerman pers. obs.
Boraginaceae	*Alkanna orientalis*	Yes	*Anthophora pauperata*	Yes	[S2]
Boraginaceae	*Mertensia ciliata*	Yes	*Bombus bifarius, B. flavifrons, B. mixtus*	Yes	D. Papaj and K. Mauerman pers. comm.
Boraginaceae	*Mertensia paniculata*	Yes	*Bombus mixtus, B. frigidus, Bombus spp.*	Yes	[S3], A. Russell pers. obs.
Boraginaceae	*Symphytum officinale*	Yes	*Bombus pascuorum*	Yes	[S4]
Boraginaceae	*Phacelia*	Yes	*Anthophora urbana*	Unknown*	[14,23]

	tanacetifolia				
Calophyllaceae	*Kielmeyera coriacea*	Yes	*Augochloropsis spp., Exomalopsis fulvofasciata, Xylocopa frontalis, X. hirsutissima*	Yes	[S5]
Calophyllaceae	*Kielmeyera speciosa*	Yes	*Augochloropsis spp., Exomalopsis fulvofasciata, Xylocopa frontalis, X. hirsutissima*	Yes	[S5]
Clusiaceae	*Clusia spp.*	Yes	*Augocloropsis spp.*	Yes	[S6]
Commelinaceae	*Tradescantia pallida*	Yes	*Anthophora spp.*	Yes	A. Russell pers. obs.
Cucurbitaceae	*Cucurbita foetidissima*	Yes	*Xenoglossa angustior*	Yes	[23]
Ericaceae	*Diospyros virginiana*	No	*Bombus impatiens, B. vagans*	Unknown*	C. Switzer pers. comm.
Fabaceae	*Astragalus spp.*	Yes	*Eucera spp.*	Yes	Z. Portman pers. comm.
Fabaceae	*Desmanthus cooleyi*	Yes	*Protoxaea gloriosa*	Unknown	[23]
Fabaceae	*Lupinus spp.*	No	*Bombus spp.*	Yes‡	K. Mauerman pers. comm.
Fabaceae	*Lupinus lepidus var. sellus*	No	*Bombus vosnesenskii*	Yes‡	J. Francis pers. comm.
Fabaceae	*Trifolium spp.*	Yes	*Bombus impatiens*	Unknown*	C. Switzer pers. comm.
Fabaceae	*Vicia spp.*	Yes	*Bombus impatiens*	Unknown*	C. Switzer pers. comm.
Fabaceae	*Coronilla varia*	No	*Bombus impatiens*	Unknown*	C. Switzer pers. comm.
Fabaceae	*Swartzia apetala*	Yes	*Bombus spp.*	Yes	[S7]
Fabaceae	*Swartzia pickelii*	Yes	*Bombus spp.*	Yes	[S8]
Fabaceae	*Lespedeza bicolor 'Natob Strain'*	No	*Bombus bimaculatus*	Unknown*	C. Switzer pers. comm.
Hypericaceae	*Hypericum 'Hidcote'*	Yes	*Bombus affinis, B. bimaculatus, B. griseocollis, B. impatiens, B.*	Yes	C. Switzer pers. comm.

			perplexus		
Lamiaceae	*Stachys recta*	Yes	*Anthophora furcata, A. quadrimaculata, Bombus pascuorum, B. terrestris, Rophites algirus*	Yes	[S9]
Liliaceae	*Polygonatum x hybridum*	Yes	*Bombus pascuorum*	Yes	[S4]
Loasaceae	*Mentzelia pumila*	Yes	*Bombus sonorus*	Yes	[S10]
Onagraceae	*Oenothera speciosa*	Yes	*Bombus impatiens*	Yes	A. Russell pers. obs.
Orchidaceae	*Thelymitra antennifera†*	Yes	*Lasioglossum spp.*	Yes	[S11]
Orchidaceae	*Thelymitra aristata†*	Yes	*Lasioglossum spp.*	Yes	[S11]
Orchidaceae	*Thelymitra nuda†*	Yes	*Lasioglossum spp.*	Yes	[S11]
Orobanchaceae	*Castelleja spp.*	Yes	*Bombus sps*	Yes	D. Papaj and K. Mauerman pers. comm.
Orobanchaceae	*Melampyrum pratense*	Yes	*Bombus lucorum, Megachile willughbiella*	Yes	[S12]
Paeoniaceae	*Paeonia spp.*	Yes	*Bombus spp.*	Yes	A. Russell pers. obs.
Papaveraceae	*Papaver rhoeas*	Yes	*Bombus terrestris*	Yes	[33]
Papaveraceae	*Argemone arizonica*	Yes	*Xylocopa californica*	Yes	[23]
Papaveraceae	*Argemone spp.*	Yes	*Bombus sonorus, Xylocopa spp.*	Yes	D. Papaj pers. comm.; A. Russell pers. obs.
Plantaginaceae	*Chelone Glabra*	Yes	*Bombus vagans, Hylaeus annulatus*	Yes	[S13]
Plantaginaceae	*Penstemon cyananthus*	Yes	*Osmia brevis*	Yes	[S14]
Plantaginaceae	*Penstemon radicosus*	Yes	*Osmia brevis*		[S14]
Plantaginaceae	*Penstemon strictus*	Yes	*Bombus nevadensis, Osmia brevis*	Yes	[S14]

Ranunculaceae	*Delphinium spp.*	Yes	*Bombus spp.*	Unknown*	D. Papaj and K. Mauerman pers. comm.
Ranunculaceae	*Aconitum spp.*	Yes	*Bombus spp.*	Yes	K. Mauerman pers. comm.
Ranunculaceae	*Aquilegia caerulea*	Yes	*Bombus spp.*		D. Papaj pers. comm.
Ranunculaceae	*Aquilegia chrysantha*	Yes	*Bombus occidentalis*	Unknown	[24]
Ranunculaceae	*Aquilegia formosa*	Yes	*Bombus spp.*	Yes	A. Russell pers. obs.
Ranunculaceae	*Cimicifuga arizonica*	Yes	*Bombus huntii, Bombus occidentalis*	Yes	[24]
Rosaceae	*Potentilla recta*	Yes	*Bombus ternarius, B. terricola*	Yes	[S15]
Rosaceae	*Potentilla gracilis*	Yes	*Bombus vosnesenskii*	Yes	J. Francis pers. comm.
Rosaceae	*Prunus dulcis*	Yes	*Bombus spp.*	Yes	[S16]
Rosaceae	*Fallugia paradoxa*	Yes	*Bombus pennsylvanicus*	Yes	[23]
Rosaceae	*Rosa 'Bucbi'*	Yes	*Bombus bimaculatus, B. impatiens*	Yes	C. Switzer pers. comm.
Rosaceae	*Rosa californica*	Yes	*Bombus edwardsii, B. vosnenskii*	Yes	[14,S1]
Rosaceae	*Rosa multiflora*	Yes	*Bombus bimaculatus, B. impatiens*	Yes	C. Switzer pers. comm.
Rosaceae	*Rosa nitida*	Yes	*Bombus ternarius*	Yes	[S15]
Rosaceae	*Rubus odoratus*	Yes	*Bombus bimaculatus, B. impatiens*	Yes	C. Switzer pers. comm.
Rosaceae	*Rosa rugosa*	Yes	*Bombus terrestris*	Yes	[S17]
Rosaceae	*Rosa virginiana*	Yes	*Bombus pennsylvanicus*	Yes	[14]
Rosaceae	*Rubus parviflorus*	Yes	*Bombus occidentalis*	Yes	[14]
Solanaceae	*Physalis philadelphica*	Yes	*Bombus impatiens*	Yes	C. Switzer pers. comm.

Solanaceae	*Physalis longifolia*	Yes	*Colletes latitarsis*	Yes	[S18]
Theaceae	*Stewartia sinensis*	Yes	*Bombus perplexus*	Yes	C. Switzer pers. comm.
Verbenaceae	*Callicarpa cathayana*	Yes	*Bombus impatiens*	Yes	C. Switzer pers. comm.
Verbenaceae	*Callicarpa dichotoma*	Yes	*Bombus bimaculatus, B. impatiens*	Yes	C. Switzer pers. comm.
Verbenaceae	*Callicarpa japonica*	Yes	*Bombus impatiens*	Yes	C. Switzer pers. comm.
Zygophyllaceae	*Kallstroemia grandiflora*	Yes	*Bombus impatiens*	Yes	A. Russell pers. obs.
Zygophyllaceae	*Guaiacum coulteri*	Yes	*Anthophora spp.*	Yes	A. Russell pers. obs.

List comprises 26 families, 47 genera, and 73 species of angiosperms
**Possible that bee sonicates to push deeper into the flower to access nectar?*
†Rewardless
‡Pushes open banner petal and then sonicates anthers

Figure S1

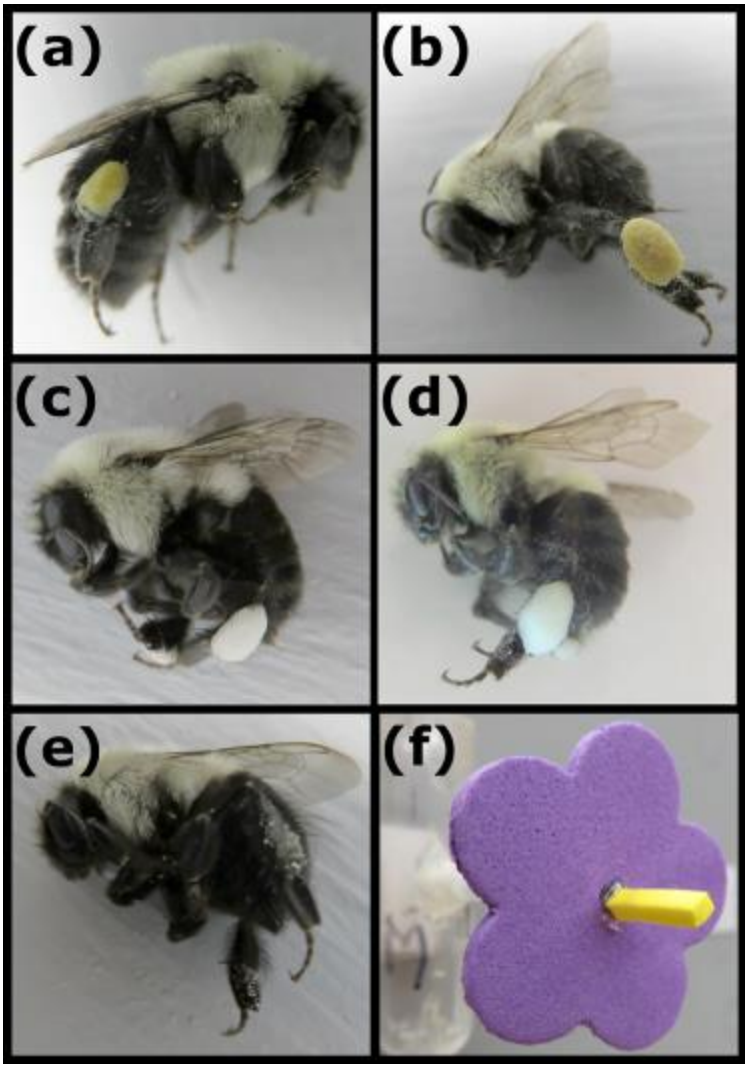


Figure S1, related to Figures 2,4. Representative *Bombus impatiens* foragers that had foraged from *Begonia descoleana* or *Begonia hybrida* flowers supplemented with pollen, cellulose powder, or plastic microspheres. Corbicular loads on the hind legs of bees that collected (a) *B. descoleana* pollen; (b) pistachio pollen, (c) 20µ plastic microspheres, (d) 20µ cellulose particles, and (e) 180µ microcrystalline cellulose particles. Bees attempted to collect large (180µ) microcrystalline cellulose particles, although they were less successful at collecting it, compared to the small (20µ) cellulose particles. All pollen loads were wetted with nectar by the bees. (f) Non-poricidal surrogate (foam) flower.

Figure S2

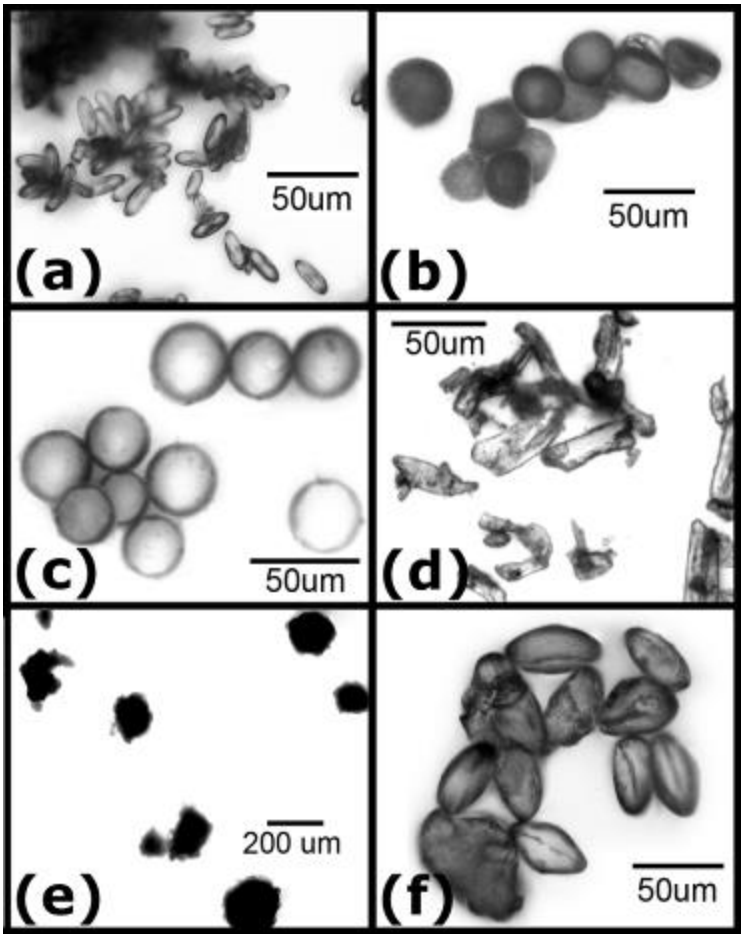


Figure S2, related to Figures 2,4. Shape and size of the pollen and particles used in this study. (a) *Begonia descoleana*, (b) Pistachio pollen, (c) 20µ plastic microspheres, (d) 20µ cellulose particles, (e) 180µ microcrystalline cellulose particles, and (f) *Prunus avium* pollen. (a-d, f) imaged at 63x and (e) imaged at 40x.

Figure S3

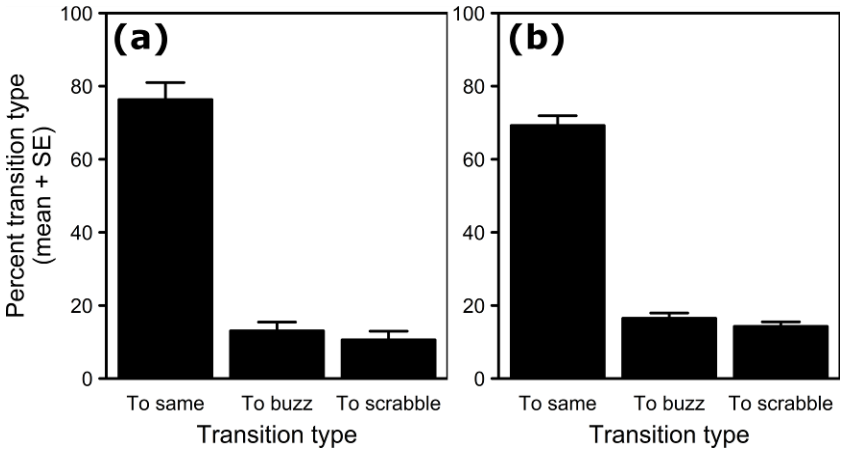


Figure S3, related to Figure 2. Mean percentage of landings (± SE) by *Bombus impatiens* that involved bees either staying with the same collection behavior or switching between collection routines from one to the next floral visit. Bees either switched from using sonication on one visit to using scrabbling on the next visit ('to scrabble'), from using scrabbling on one visit to using sonication on the next visit ('to buzz'), or did not switch collection behaviors from one to the next visit ('to same'). (a) Bees foraging on *Begonia descoleana*; (b) Bees foraging on *B. hybrida* supplemented with 1mg of pistachio pollen. N = 9 and 7 bees for *B. descoleana* and *B. hybrida* treatments, respectively; data from experiment 1.

Figure S4

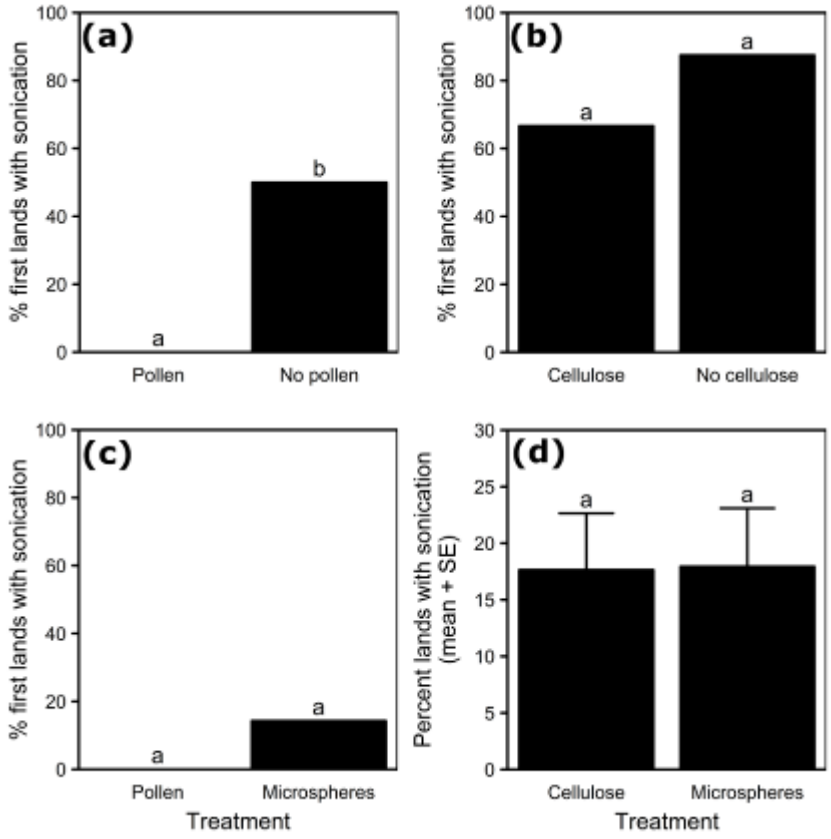


Figure S4, related to Figures 2,4. Percentage of the first landing and mean percentage of all landings (± SE) by *Bombus impatiens* resulting in sonication in treatments where the availability of pollen, cellulose particles, or plastic microspheres presented by *Begonia hybrida* was varied. Percentage of the first landing of naïve bees resulting in sonication on (a) pollenless flowers or 1.5mg pollen-supplemented flowers; (b) pollenless flowers or 2mg large (180µ diameter) cellulose particles-supplemented flowers; (c) 2mg pollen-supplemented flowers or 2mg (20µ diameter) plastic microspheres-supplemented flowers. (d) Mean percentage of landings (± SE) resulting in sonication on 2mg pollen-supplemented flowers or 2mg (20µ diameter) plastic microspheres-supplemented flowers. Bees never scrabbled on bare flowers. *N* = 12, 11 bees for the *B. hybrida* pollenless and pollen-supplemented treatment, respectively; data from experiment 1. *N* = 7 bees for both

the *B. hybrida* pollen and plastic microsphere-supplemented treatment; pollen-supplemented treatment data from experiment 1. Different letters above bars indicate significant differences at $p<0.05$ according to tests of equal proportions or a *t*-test.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Bees

We used 154 workers from eight commercially obtained (Koppert Biological Systems, Howell, MI, USA) colonies of the bumble bee *Bombus impatiens* Cresson in laboratory experiments conducted between June 2014 and April 2016. Bumble bees are globally distributed generalist pollinators, and species such as *B. impatiens* forage from hundreds of angiosperm species [19]. We used approximately equal numbers of bees from each colony and each treatment. We allowed bees to forage daily for sucrose and pollen in arenas constructed of plywood (LxWxH 82 x 60 x 60 cm). The arenas were lit from above by 40W 4400 lumen LED lights (2x2 LED Ultra Thin Panel; 5000K Cool White, James Industry) set to a 14 h :10 h light : dark cycle. Colonies had access to *ad libitum* 2M sucrose solution and pulverized honey bee-collected pollen (Koppert Biological Systems, MI, USA) within the foraging arena. Sucrose solution was dispensed via braided cotton wicks that extended into vials. Pollen was dispensed via custom-made feeders constructed of chenille fibers glued within 40 dram vials [13]. Bees always scrabbled for this pollen; further, of bees naïve to pollen foraging that were observed on their first few visits to feeders, none sonicated. Bees were also naïve to the pure (i.e.

hand-collected) pollen used in experiments: these pure pollen types were not present in the honey bee-collected pollen (Kim Skyrm, Koppert Biological Systems, pers. comm.), which moreover does not resemble pure pollen, as honey bee-collected pollen is adulterated with up to 60% by mass regurgitated crop sugars [13].

Plants and Flowers

We used freshly clipped flowers with mature anthers from eight poricidal *Solanum houstonii* Martyn (synonym, *S. tridynamum*) plants, two non-poricidal *Begonia x hybrida* ('Dragonwing'), and 10 non-poricidal *Begonia descoleana* L. B. Sm. and B. G. Schub. plants raised in a university greenhouse and fertilized weekly (Miracle Gro, NPK = 15-30-15). The anthers of *B. hybrida* are sterile (pollenless) and made it possible to precisely control pollen cues presented to bees. Conversely, *B. descoleana*, one of the species crossed to make the *B. hybrida* hybrid, is fertile and presents pollen on its anthers (its only floral reward). Likewise, *S. houstonii* only offers pollen to its pollinators. Flowers across plants were approximately equally represented in a given trial. We used approximately 2000 flowers in experiments.

General Experimental Protocol

All trials took place in a foraging arena (LxWxH, 82cm x 60cm x 60cm) painted gray on floor and sides. In trials, freshly clipped flowers were displayed horizontally (their natural orientation) on custom-built water tubes (see [S20]), to prevent desiccation. The water tubes were Velcro-mounted on the arena wall, facing the flight chamber's nest entrance. Flowers were arranged on the wall in a 3 x 3 Cartesian grid with each water tube spaced 7

cm apart in the horizontal and vertical axes of the grid. 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 (for experiment 1, the sub-experiments) in time to control for effects of day and time of day on behavior.

To initiate a trial, a single flower-naïve individual was introduced into the arena. Bees readily visited all types of flowers, live and artificial. We recorded landings made by the bee on the flowers. A landing was defined as taking place when a bee touched the flower with at least three legs simultaneously. Three types of landings were noted: landings with sonication buzzes, landings with only scrabbling, and landings without scrabbling or sonication buzzes. On rare occasions in experiment 1 and 3 only, bees switched from scrabbling to sonicating on the same landing: these rare landings were also classified as landings with sonication buzzes. Sonication buzzes were identified by their distinctive sound and the stereotyped posture of the bees on the flowers (see [19] for extended description) and occurred only after a bee had landed. Scrabbling involved the bee manipulating the anthers with the mandibles and legs (see [13] for videos and extended description). Virtually all flower visits where flowers presented pollen, small 20μ diameter cellulose powder, or 20μ diameter plastic microspheres (Figure S2) involved collection attempts (either by sonicating or scrabbling). Bees never scrabbled when the anthers (surrogate or live) were bare or presented large 180μ diameter cellulose powder. We tracked whether bees landed on previously unvisited flowers or on previously visited flowers to allow comparison of behavior between these two categories. Bees nearly always visited all flowers in an array at least once. Bees were allowed to make up to 20,

30 or 40 landings in an array (depending on experiment), after which the trial was terminated. A trial was sometimes terminated before the maximum number of landings if the bee did not forage for a period of five minutes. Most bees (71%) made the maximum number of allowed landings (bees visiting unrewarding arrays tended not to complete the maximum number of allowed landings) and all bees were included in analyses. We euthanized each bee and all its flowers after the bee completed its trial: bees were only tested once.

To facilitate recording of behavior, video for all trials was captured at 30fps 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 Lavaliere Microphone, RadioShack) attached to the center of floral arrays. A Zoom H2 Handy Recorder (ZOOM Corporation) was used to amplify and verify sonication buzzes in ongoing trials.

Experiments

Experiment 1

Here we sought to determine whether bees buzzed the flowers of non-poricidal species and the role of pollen availability in mediating this response. This experiment (composed of three sub-experiments) used 54 bees from seven colonies, and each bee was presented with an array of nine flowers. In experiment 1a we used the natural species, *B. descoleana* to characterize the normal pollen collection behavior of bees; in experiments

1b and 1c we used the pollenless hybrid, *B. hybrida*, supplemented with controlled amounts of pollen to precisely determine how pollen availability affected pollen collection behavior.

In experiment 1a, bees were each allowed 40 landings. We split bees in experiment 1b into two treatments and allowed each bee 20 landings. In one treatment, *B. hybrida* flowers were each supplemented with 1.5mg pistachio pollen (*Pistacia vera*; Pollen Collection and Sales; Lemon Cove, California, USA) added to their anthers (mean ± SE: 1.52 ± 0.02). Pollen was stored at -18 C and weighed using a Sartorius Analytic Balance (Data Weighing Systems, Inc.) to the nearest 0.1mg. Pollen was stored at -18 C and weighed using a Sartorius Analytic Balance (Data Weighing Systems, Inc.) to the nearest 0.1mg. We used pistachio pollen in experiment 1b, because it was available in large quantities, unlike *Begonia* pollen. In the other treatment, *B. hybrida* flowers were pollenless (their natural state). In experiment 1c, bees were split into three treatments and each allowed 40 landings. Each treatment varied by the quantity of added pollen: flowers presented 1, 2, or 4mg of pollen on their anthers (mean ± SE: 1.0 ± 0.01; 2.0 ± 0.01; 4.0 ± 0.01). We discarded one bee that collected pollen only five times. The amount of pistachio pollen added to flowers was within the range that live flowers contain (see [19]).

Experiment 2

Here we wanted to determine whether chemical anther cues of non-poricidal *B. hybrida* and poricidal *S. houstonii* elicited sonication by bees. To this end, we used surrogate

flowers made with real corollas and surrogate foam anthers. Use of the live flower's corolla allowed us to assess whether an anther extract applied to the surrogate anthers alone elicited sonication. This experiment used 22 bees from five colonies.

We made surrogate flowers by cutting off and discarding the stamens from the corollas of flowers. Pure pentane or a pentane anther extract was applied to Yellow Fibrecraft Foam (Jo-Ann Stores, LLC.), cut into cuboids (LxWxH 1.4 x 0.2 x 0.2cm). These surrogate anthers were hot-glued to the corollas. Surrogate flowers were arranged in a 3 x 3 grid without a central flower (eight total targets), with pentane control and extract-treated targets alternated by position. Details below. We split bees into two treatments and allowed each bee 30 landings. Arrays and extracts were made from *B. hybrida* in one treatment and from *S. houstonii* in the other treatment.

To prepare pentane anther extracts, 1.5 mL of Pentane HPLC Grade (Fisher Scientific Company, LLC.) was added to a sterile 4 dram glass vial (BioQuip Products, Inc CA, USA). Two types of anther extracts were made for this study: extracts made from 120 flowers of *Begonia hybrida* or from 60 flowers of *Solanum houstonii*. For both types of extracts, the anthers of 20 flowers (~480 *B. hybrida* anthers or 100 *S. houstonii* anthers) were excised at the filament base and submerged within the pentane for 5 minutes. Anthers were removed from the vial with sterile forceps. An amount of pentane equal to that which had evaporated when the anthers were removed (typically ~0.2mL) was added to the vial. These steps were repeated until the pentane extract contained, in the case of the *B. hybrida* extract, an equivalent of 120 flowers' anthers (~2880 anthers), and in the

case of the *S. houstonii* extract, an equivalent of 60 flowers' anthers (~600 anthers) in 1.0mL of solvent. These extracts were transferred to separate sterile 2.0mL amber autosampler vials (Thermo Fisher Scientific Inc.). Prepared extracts were stored at -18 C.

To prepare surrogate flowers, Yellow Fibrecraft Foam (Jo-Ann Stores, LLC.) was cut into cuboids (LxWxH 1.4 x 0.2 x 0.2cm) that were about the length of the *Solanum* stamens, but several times the length of *Begonia* stamens. 0.1mL pure pentane or anther extract were applied to each foam surrogate anther via 1mL Pyrex disposable serological pipettes (Sigma-Aldrich Co. LLC.). When the solvent had evaporated (the foam conveniently turns white for ~1 second upon evaporating), the surrogate was ready to be glued in place. The anthers of flowers were excised and an extract-treated surrogate hot-glued at this spot (Figure S1f). This order is critical, as pentane solvent otherwise destroys floral tissue (A. Russell, pers. obs.). We discarded surrogate flowers after 30 minutes if unvisited, in case the anther extract lost its attractiveness to the bees with time.

Experiment 3

We investigated whether mechanical stimulation by odorless particles, similar in size to pollen, mediated pollen collection behavior similarly to pollen. This experiment (composed of two sub-experiments) used *B. hybrida* and 38 bees from two colonies; each bee was presented with an array of nine flowers.

In experiment 1a, bees were allocated to each of three treatment groups and each bee allowed 40 landings. Each treatment varied by the quantity of supplemental small

cellulose powder (20µ Cellulose Microcrystalline Powder; Sigma Aldrich, St. Louis, Missouri, USA): flowers presented 1, 2, or 4mg of cellulose powder on their anthers (mean ± SE: 0.98 ± 0.03; 2.0 ± 0.02; 3.99 ± 0.02). We discarded one bee that landed on flowers, but did not collect any cellulose.

We examined pollen and cellulose powder under a compound microscope to determine whether cellulose powder physically resembled pollen, and thus was a good proxy. We found that unlike pistachio, cherry (*Prunus avium*; Pollen Collection and Sales; Lemon Cove, California, USA), or *Begonia* pollen, 20µ diameter cellulose powder (180µ diameter microcrystalline cellulose powder less so) were not of uniform size or shape (Figure S2). However, in experiment 1b, assays comparing foraging behavior on arrays of flowers offering either 2mg 20µ cellulose powder (using concurrently tested bees from experiment 1a) or 2mg uniformly shaped and sized 20µ diameter polystyrene microspheres (Figure S2c; 20µ Polystyrene DVB Microspheres; Thermo Scientific, Fremont, CA, USA) confirmed using cellulose did not affect patterns of behavior (Figure 4,S4d; Welch two sample *t*-test: proportion of visits that involved sonication, 20µ cellulose x 20µ microspheres, $t_{11.991} = -0.0428$, $P = 0.967$). We discarded two bees which landed on flowers, but did not collect microspheres or cellulose, respectively.

Experiment 4

Here we wanted to find out whether pollen collection behavior was mediated by pollen-sized particles specifically. This experiment used *B. hybrida* and 17 bees from two colonies; each bee was presented with an array of nine flowers.

Bees were allocated into each of two treatments and each bee allowed 40 landings. In one treatment, flowers each had 2mg of 180μ diameter microcrystalline cellulose (Avicel PH-200 NF; FMC Corporation, Philadelphia, PA, USA) added to their anthers (mean ± SE: 2.0 ± 0.02). These cellulose particles are larger than typical pollen offered by plants and collected by bees (mean diameter: 34μ; [25]). In the other treatment flowers were bare. We discarded one bee that made only two landings.

Experiment 5

Here we investigated whether bees that collected pollen from non-poricidal flowers by sonicating were able to collect it at a faster rate than those that collected pollen by scrabbling. This experiment used artificial flowers and 23 bees from two colonies. We used artificial flowers to control the amount of pollen presented and whether bees would sonicate or scrabble.

Bees were allocated into each of two treatments: in one treatment bees only scrabbled for pollen and in the other treatment bees only sonicated for pollen. Each bee was allowed two landings to each artificial flower (8 pollen collecting landings per bee), whereupon the flower was removed from the foraging arena. While removing the flower the bee continued to visit flowers and did not exhibit signs of being threatened by our activity, such as aggressive behavior or attempts to escape from the arena.

The artificial flower's corolla (diameter: 2.8cm) was made from purple Fibrecraft Foam, cut with a puncher (Medium Plum Blossom; Punch Bunch Inc., CO, USA). The surrogate anthers were of the same design used in experiment 2 and were hot-glued onto the center of the corolla, such that two of the rectangular surfaces faced up at an angle simultaneously (Figure 5a). Cherry (mean mg per anther ± SE: 0.63 ± 0.05, N = 15 measurements) was spread evenly onto those two surfaces.

Flower-naïve bees scrabbled for pure cherry pollen on artificial flowers and never sonicated. To ensure that bees in the comparison treatment only ever sonicated on artificial flowers, we trained bees to sonicate in response to a floral odor, prior to visiting artificial flowers. In this training, bees were allowed to make eight rewarding visits to an array of four poricidal *S. houstonii* flowers (pollen is their only floral reward); sonication is the only behavior these bees use to extract pollen from the poricidal anthers. After this training, bees were labeled with unique color combinations of acrylic paint, and returned to their colony. After depositing its pollen loads the bee was tested by allowing it to collect cherry pollen from artificial flowers treated with *S. houstonii* anther extract. Bees tested in this way always sonicated to collect pollen. We discarded one bee that completed the training phase, but did not visit the surrogate flowers in the testing phase. Training compelled bees to sonicate, but should not have made bees more effective sonicators (and thus more efficient pollen collectors than if they had never sonicated previously): sonication is fully effective at first expression and modified little with experience (see [19] for details). Bees had extensive experience scrabbling, but both set of bees were naïve to foraging on surrogate anthers and pure pollen.

To calculate the rate of pollen collection per bee we divided the amount of cherry pollen collected by the total time on the flower (defined by the start of the flower visit until the end of a visit, summed across all eight bee landings). Total time on flower was estimated from video footage viewed frame-by-frame with Avidemux software (fixounet@free.fr). The end of a visit was defined as the first video frame in which the bee no longer contacted the flower with its legs. To determine the amount of pollen collected, we euthanized each bee immediately after it completed its trial and removed and weighed its pollen load.

Data Analyses

All data were analyzed using R v.3.2.0 [S21].

Variables being analyzed were a composite of each bee's responses; specifically they were proportion variables. We analyzed differences in the proportion of landings that included sonications, in the proportion of landings with sonication per flower type, and in the rate of cherry collection. When analyzing differences between two treatments (or two flower states) we used *t*-tests if assumptions of normality and equal variance were met (using Shapiro-Wilk and F tests, respectively, in the mgcv package: [S22]) or, otherwise, Wilcoxon-signed rank tests. Where we were interested in patterns across three treatments, we used one-way analyses of variance (ANOVAs) using the aov() function in R. In cases

of significant effects, we ran Tukey's post hoc test, using the TukeyHSD() function in R, to determine which pairs were significant.

SUPPLEMENTAL REFERENCES

S1. Buchmann, S.L. (1978). Vibratile ("buzz") pollination in angiosperms with poricidally dehiscent anthers. PhD thesis, University of California, Davis, CA, USA.

S2. Stone, G.N, Gilbert, F., Willmer, P., Potts, S., Semida, F., and Zalat, S. (1999). Windows of opportunity and the temporal structuring of foraging activity in a desert solitary bee. Ecol. Entomol. *24*: 208-221.

S3. Morris, W.F. (1996). Mutualism denied? Nectar-robbing bumble bees do not reduce female or male success of bluebells. Ecology. *77*, 1451-1462.

S4. Corbet, S.A., Chapman, H., and Saville, N. (1988). Vibratory pollen collection and flower form: bumble-bees on *Actinidia*, *Symphytum*, *Borago* and *Polygonatum*. Funct. Ecol. *2*, 147-155.

S5. Oliveira, P.E.A.M., and Sazima, M. (1990). Pollination biology of two species of *Kielmeyera* (*Guttiferae*) from Brazilian cerrado vegetation. Plant. Syst. Evol. *172*, 35-49.

S6. Kaminski, A.C., and Absy, M.L. (2006). Bees visitors of three species of *Clusia* (Clusiaceae) flowers in Central Amazonia. Acta. Amazonica. *36*, 259-264.

S7. Chiara Moço, M.Cd, and Pinheiro, M.C.B. (1999). Pollination ecology of *Swartzia Apetala* raddi var. *Apetala* (Leguminosae-Papilionoideae). Braz. Arch. Biol. Technol. *42*, 1-9.

S8. Machado, I.C., and Lopes, A.V. (2004). Floral traits and pollination systems in the Caatinga, a Brazilian tropical dry forest. Ann. Bot. *94*, 365-376.

S9. Müller, A. (1996). Convergent evolution of morphological specializations in Central European bee and honey wasp species as an adaptation to the uptake of pollen from nototribic flowers (Hymenoptera, *Apoidea* and *Masaridae*). Biol. J. Linnean. Soc. 57, 235-252.

S10. Linsley, E.G., and Cazier, M.A. (1963). Further observations on bees which take pollen plants of the genus *Solanum*. *Pan-Pac Entomol. 39*, 1-18.

S11. Bernhardt, P., and Burns-Balogh, P. (1986). Floral mimesis in *Thylmitra nuda* (Orchidaceae). Plant. Syst. Evol. *151*, 187-202.

S12. Meidell, O. (1944). Notes on the pollination of *Melampyrum pratense* and the "honeystealing" of bumblebees and bees. Bergens Museums aarbok *11*, 5-11.

S13. Richardson, L.L., and Irwin, R.E. 2015. Pollination ecology and floral visitor spectrum of turtlehead (*Chelone Glabra* L.; Plantaginaceae). J. Pollinat. Ecol. *17*, 132-144.

S14. Cane, J.H. (2014). The oligolectic bee *Osmia brevis* sonicates *Penstemon* flowers for pollen: a newly documented behavior for the Megachilidae. Apidologie. *45*, 678-684.

S15. Heinrich, B. (1976). The foraging specializations of individual bumblebees. Ecol. Monogr. *46*, 105-128.

S16. Thomson, J.D., and Goodell, K. (2001). Pollen removal and deposition by honeybee and bumblebee visitors to apple and almond flowers. J. App. Ecol. *38*, 1032-1044.

S17. Dobson, H.E.M., Danielson, E.M., and Wesep, I.D.V. (1999). Pollen odor chemicals as modulators of bumble bee foraging on *Rosa rugosa* Thunb. (Rosaceae). Plant Species Biol. *14*, 153-166.

S18. Paine, K.C., and Roulston, T.H. (2012). Thieves or friends: are specialist bees more efficient at removing pollen than generalists? Conference paper, ESA Annual Convention 2012.

S19. Laverty, T.M., and Plowright, R.C. (1988). Flower handling by bumblebees: a comparison of specialists and generalists. Anim. Behav. *36*, 733-740.

S20. Russell, A.L., Golden, R.E., Leonard, A.S., and Papaj, D.R. (2016). Bees learn preferences for plant species that offer only pollen as a reward. Behav. Ecol. *27*, 731-740.

S21. R Development Core Team. 2010. R: a language and environment for statistical computing. Vienna (Austria): R Foundation for Statistical Computing.

S22. Wood, S. 2016. Mixed GAM computation vehicle with GCV/AIC/REML smoothnessestimation (R package version 1.9e9) https://stat.ethz.ch/R-manual/R-devel/library/mgcv/html/mgcv-package.html (accessed October 2016).