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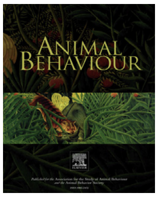

Concealed floral rewards and the role of experience in floral sonication by bees

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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.

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, Thomson, & Waser, 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. Gegear & Laverty, 1995; Laverty, 1994). Cognitive constraints associated with learning, forgetting and relearning these motor routines are thought to discourage pollinators from switching back and forth among plant species (Chittka et al., 1999; Gegear & Laverty, 1995; Lewis, 1993). Pollinators exhibiting floral fidelity provide direct benefits to the plant in terms of reduced pollen wastage and foreign pollen interference (Gegear & Laverty, 1995; Waser, 1986). 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 (Chittka et al., 1999; Lewis, 1993; Plowright & Laverty, 1984).

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 (Kevan & Baker, 1983; Nicolson & van Wyk, 2011; Simpson & Neff, 1981). 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, &

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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 (Buchmann & Cane, 1989; Pellmyr, 1988). 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, Hogendoorn, Ravi, & Combes, 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 & Lengoc, 1993; Morgan, Whitehorn, Lye, & Vallejo-Marin, 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.

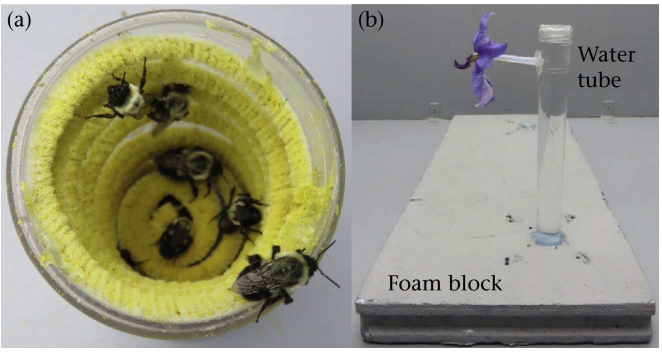

Figure 1. (a) Chenille stem feeder loaded with honeybee-collected pollen (reproduced from Russell & Papaj, 2016). (b) Grey foam block used to mount water tubes, from which flowers were displayed. Each water tube held only a single flower. Flowers were displayed horizontally and glued into the water tube,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

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, Lunau, & Schlindwein, 2011; Nunes-Silva, Hnrcir, Shipp, Imperatriz-Fonseca, & Kevan, 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, Cox, & Vallejo-Marín, 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 the release of pollen. 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

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.

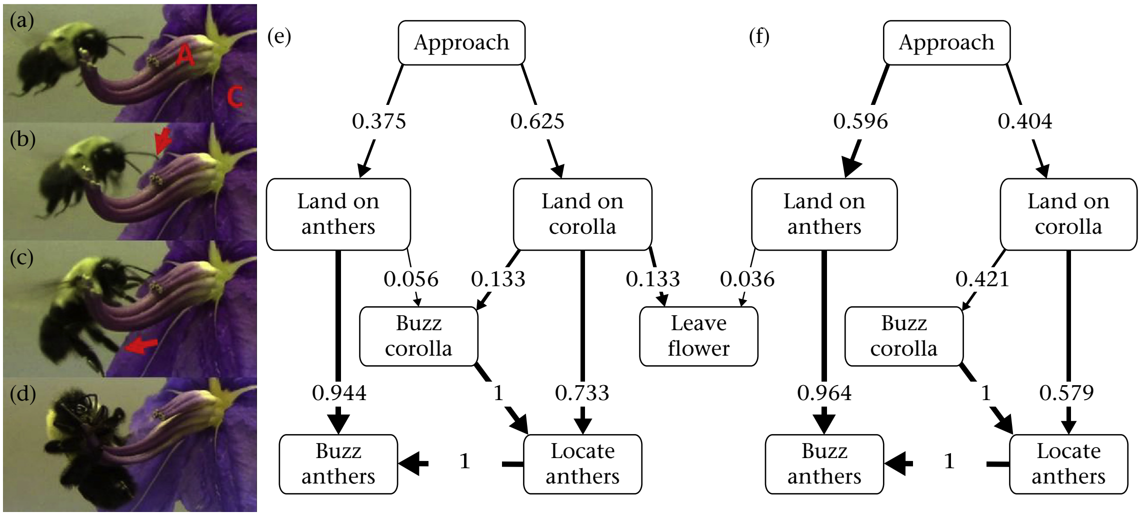

Figure 2. (a–d) Key phases in the first floral visit up until the first buzz or until the bee left the flower: (a) approaching the flower, (b) antennal contact with the flower indicated with red arrow, (c) landing indicated with red arrow, (d) buzzing anthers. Anthers indicated by the red letter 'A'; 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).

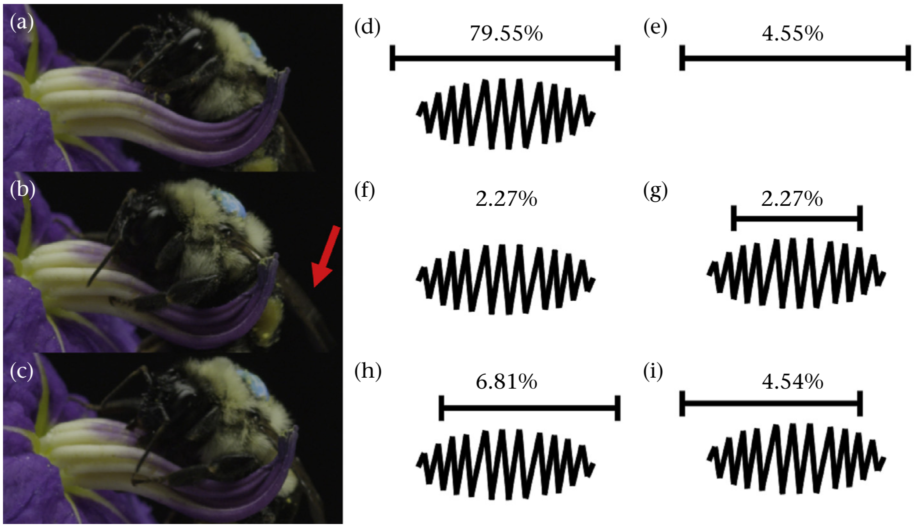

Figure 3. (a–c) Key phases in the floral sonication motor routine: (a) antennal contact and clamping the mandibles on floral tissue, (b) sonicating; blur caused by vibration of wings during buzz indicated with red arrow, (c) releasing mandibles. (d–i) The six kinds of floral sonication motor routines 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/bite to find an analysable sequence ± SE: 3.59 ± 0.52, $N = 44$ bees).

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, 2010). 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, Maechler, Bolker, & Walker, 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 and 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, 1991; Evangelista, Kraft, Dacke, Reinhard, & Srinivasan, 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 f). 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 and 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

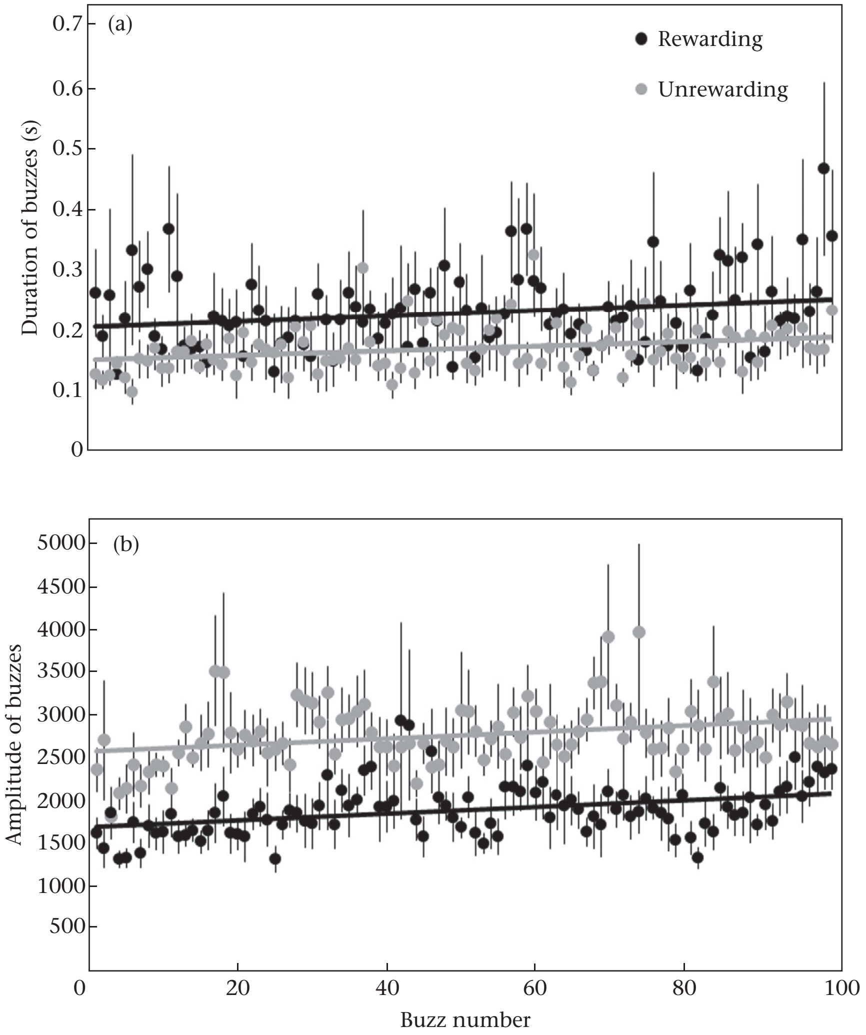

Figure 4. Mean ± SE (a) duration and (b) amplitude of sonications for the first 100 buzzes (rewarding treatment: $N = 6$ bees; unrewarding treatment: $N = 7$ bees). Although analyses were performed on log-transformed data, means and SEs are shown for the untransformed data.

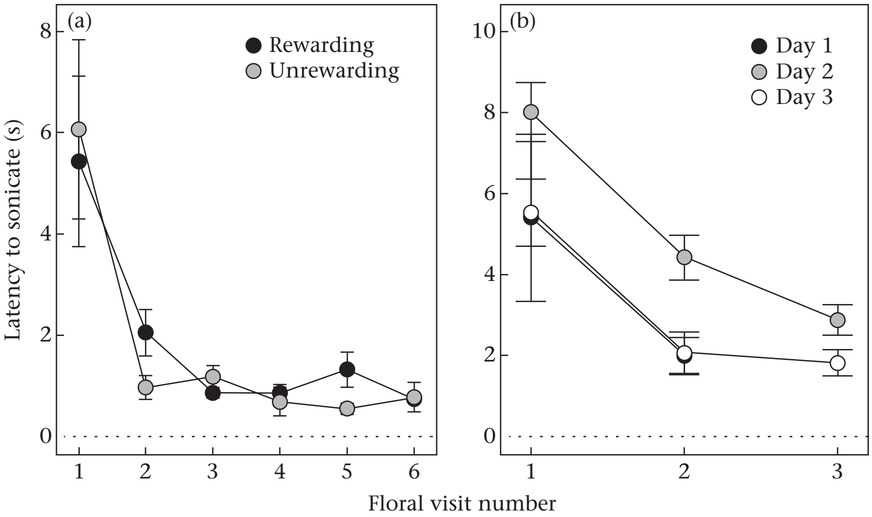

Figure 5. (a) Mean ± SE latency to sonicate on the first six floral visits to a rewarding flower ($N = 15$ bees) and an unrewarding flower ($N = 13$ bees). (b) Mean ± SE latency to sonicate on rewarding flowers for the first three floral visits and across days ($N = 10$ bees).

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 f).

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 (Chittka et al., 1999; Gegear & Laverty, 2005; Lewis, 1993). 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, Golden, Leonard, & Papaj, 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') (Gegear & Laverty, 1998; Lewis, 1986; Woodward & Laverty, 1992). 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 (Gegear & Laverty, 1995; Laverty, 1994; Woodward & Laverty, 1992). 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 (Edlund, Swanson, & Preuss, 2004; Gottsberger & Silberbauer-Gottsberger, 1988; Johnson & McCormick, 2001; Zhang, Yang, & Duan, 2014). Poricidal morphology may also restrict the amount of pollen a forager can remove for its own use (Castellanos, Wilson, Keller, Wolfe, & Thomson, 2006; Hargreaves, Harder, & Johnson, 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, Alexandersson, & Johnson, 2009; Newman, Manning, & Anderson, 2014). For instance, many poricidal species have evolved stamens divided into ‘feeding’ and ‘pollinating’ functions (i.e. heteranthery), which further reduces pollen wastage (Li et al., 2015; Vallejo-Marín, Da Silva, Sargent, & Barrett, 2010).

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, Papaj, & Leonard, 2015, 2016; Russell et al., 2015; but see also Arenas & Farina, 2012; Nicholls & Hempel 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 (Gegear & Laverty, 1995, 1998; Lewis, 1986). 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 (Chittka et al., 1999; Gegear & Laverty, 1995; Waser, 1986).

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.

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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.anbehav.2016.07.024.

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