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Individually recognizable scent marks on flowers made by a solitary bee

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The marking of flowers with ephemeral scent is an underappreciated but vital element in the foraging behaviour of social bees. Using observational and experimental data, we tested whether a solitary bee (female *Anthophora plumipes*) uses scent marking while foraging on flowers of *Cerinthe major* in Portugal. Females used scent marks with at least two components that differed in their volatility and, furthermore, recognized the marks of different individuals. A very short-term component (<3 min) was attractive, resulting in the observed high level of immediate revisits: this component appeared to be adjusted according to the foraging needs of the moment. A longer-term component (<30 min) was initially repellent and matched the rate of nectar renewal; it, or the response to it, also appeared to be adjusted to the perceived level of nectar reward. There may be even longer-term effects associated with the specific foraging areas of individual bees. Observed differences in the way in which individuals responded to scent marks indicate that they may play a role as part of a dominance or exclusion mechanism among females.

Energetic considerations in foraging strategies have contributed greatly towards understanding how animals forage for food (Pyke 1984; Stephens & Krebs 1986), particularly in flower-visiting bees (e.g. Pyke 1980; Cresswell 1990; Dukas & Real 1993). Most models of foraging behaviour applied to bees assume that they update estimates of gain rates continuously, usually based on the last one or two flowers visited, together with what they perceive of the nearby resource environment.

However, subsequent to these tests of optimal foraging theory, we now know that other aspects of foraging behaviour may be just as important, based upon more realistic models of resource availability, and some subtle behavioural features of real bees. Possingham (1989) developed a model of nectar distribution and renewal showing that if foraging is truly random, then the interarrival times at flowers should have an exponential distribution, and the mean and variance of random samples of flowers should be the same as that encountered by a bee. Any deviations from random foraging imply that the mean crop encountered by a bee should be different from that measured by a researcher sampling flowers at random. Whether nectar renewal is linear or nonlinear with time determines the impact of systematic foraging on the mean and variance of encountered nectar, relative to random samples. If renewal is linear, no form of nonrandom foraging will increase the rate of resource acquisition. However, if the rate of renewal decreases with time (i.e. is nonlinear), then foraging more systematically will increase the mean encountered crop (see Kadmon 1992). Possingham pointed out that even if systematic foraging does not increase the rate of energy gain, a forager might forage systematically to reduce the mean standing crop, making a patch less profitable to intruders; alternatively, a risk-averse forager might benefit by reducing the variance of the encountered reward (Krebs & Kacelnik 1991). In reality, it is obvious that bees do not forage randomly, but show various forms of systematic foraging behaviour.

Possingham (1989) suggested four mechanisms of nonrandom foraging behaviour, all of which will decrease the variance/mean ratio of interarrival times at flowers: area-restricted searching, identification and rejection of poorly

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rewarding flowers, trapline foraging, and the systematic exploitation of a territory. All of these mechanisms occur in social bees (Heinrich 1979; Seeley 1985; Thomson et al. 1987; Cresswell 1990; Collett et al. 1993; Dukas & Real 1993). In this paper we concentrate on one of them, the identification and rejection of poorly rewarding flowers. It has long been known that social bees do this (Ferguson & Free 1979; Heinrich 1979; Free & Williams 1983; Corbet et al. 1984; Marden 1984; Weatherwax 1986; Kato 1988; Giurfa & Nuñez 1993), at least partly by using morphological cues from flowers (Duffield et al. 1993; Gonzalez et al. 1995), but also by using scent marks (Cameron 1981). Like the case of the very obvious traplining (Heinrich 1976), this feature of the behaviour of social bees has been almost completely ignored, despite many aspects of foraging theory having being tested extensively on both honeybees and bumblebees. Although authors have variously suggested that bees are able to smell (Heinrich 1979; Galen 1989), see (Thorp et al. 1975; Willmer et al. 1994) or in some other way remotely perceive the nectar rewards directly (Marden 1984), some elegant laboratory (Cameron 1981; Schmitt & Bertsch 1990; Schmitt et al. 1991) and field (Goulson et al. 1998; Stout et al. 1998; Williams 1998) experiments implicate their use of tarsal scent marks, confirming some interpretations of field observational data.

Schmitt & Bertsch's (1990) laboratory experiments on bumblebees showed that both short-term repellent and long-term attractant scent mark components were used in the exploitation of floral resources for the colony; when denied the use of attractant marks, their rate of energy gain decreased significantly. Recent detailed field studies of scent marking have shown that the scent marks appear not to be specific even to different bumblebee species, let alone to individual bees. However, bumblebees and honeybees do not appear to respond to each other's scent marks, implying that at this level they are distinguishable (Goulson et al. 1998; Stout et al. 1998; Williams 1998). Individuals from the same colony of these social bees do not compete against one another, and hence one might predict colony-level recognition of scent marks.

Dukas & Real (1991) suggested that solitary bees are less able to learn or to memorize floral features than social bees. If true, this might imply that solitary bees were less able or unable to operate the same mechanisms of non-random foraging. However, the use of scent marks has been suspected in solitary bees (Frankie & Vinson 1977; Gilbert et al. 1991; Kadmon et al. 1991; Kadmon 1992; Kadmon & Shmida 1992), but is poorly studied. Therefore we designed the present study to test whether scent marking is part of the foraging repertoire of solitary as well as social bees. Specifically we tested whether female *Anthophora plumipes* bees mark flowers of honeywort, *Cerinthe major*, with scent during their foraging bouts.

Female solitary bees forage only for their own offspring, and compete against one another and against social bees for the available floral resources. We therefore predicted that, in their use of scent marking in their foraging behaviour, solitary bees would use marks of two types, or a single mark with two components. Some flowers would be marked for special attention with a long-term attractive mark, either because these flowers lay within a territory, or conveniently on a trapline (Thomson et al. 1982, 1987; Thomson 1988), or because the flowers were highly rewarding owing to their relatively high secretion rates (cf. Gilbert et al. 1991). This mark would decay only very slowly, and would probably be individually recognizable (for identifying one's own trapline; females obtain no benefit if others can also identify the high-rewarding flowers), or provoke at least some sort of differentiation between classes of females (e.g. dominant/subordinate in territorial marking). In the second type of scent mark, recently visited flowers would be marked with a repellent scent mark whose decay rate matched the renewal rate of the resource: this mark would therefore decay relatively quickly. Because the energetics of foraging for oneself are different from foraging for a colony (cf. Schmid-Hempel et al. 1985), we predicted further that the rate of decay of such a mark would be longer than those reported for social bees. Such a mark may or may not be individually recognizable.

We present here observational and experimental data testing these predictions. We tested for three components: the presence of a scent mark, its decay with time, and the recognition of individual scent marks.

METHODS

We carried out this study during April 1994–2000 at two sites: the Quinta da São Pedro Field Station, Sobreda di Caparica, near Lisbon, Portugal (38°39′N, 9°11′W); and the 'ditch site', 5 km from the Quinta, on the sides of a drainage ditch by the road between Charneca and Caparica where there were groups of *Cerinthe* plants. Where cited, mean values are given as ± 1 SE, and all times are cited in European time (GMT+1 h), current in Portugal during most of the study.

Study Species

We studied the foraging of female *A. plumipes* (Pallas) (Hymenoptera: Anthophoridae) on a white Portuguese form of honeywort, *C. major* (L.) subsp. *gymnandra* Gasparrini 1842 (Boraginaceae). The study period covered most of the middle of the flowering period of the plant. In most years there were 20–60 plants and 200–500 inflorescences in the Quinta study site, all growing in an area about 10×1 m: in the ditch site there were large numbers of plants and flowers in some years, few in other years. We know of no other *Cerinthe* plants for at least 5 km from these sites in any direction. Flowers of *Cerinthe* are tubular and long, with the nectar hidden deep within, accessible only to very long-tongued bees: we have never seen signs of holes bitten in the corolla sides by nectar robbers. The inflorescence of *Cerinthe* is very characteristic of the Boraginaceae. It is a determinate scorpioid cincinnus that uncoils progressively as the flowers open. Each plant of the study had between 1 and 40 such inflorescences. At any one time there are only two *Cerinthe* flowers open on an inflorescence: if a third is present it has already abscized at the base and drops at a

touch. Bees almost never visit the older of the two flowers (0.6% of visits), but confine their visits to the younger: thus there is effectively only a single flower per inflorescence (see Gilbert et al. 1991). In the majority of flowers, the five anthers dehisce on the second day after opening, and flowers are presumably female from then on. Each flower eventually produces between zero and four seeds. *Cerinthe* appears to be a buzz-pollinated flower.

Visitors to the plants almost exclusively consisted of the following taxa. Females of *A. plumipes* were very common, with occasional visits from males; individuals of at least two other species of *Anthophora* (probably *fulvitarsis* and *hispanica*) were uncommon or rare; in some years there were occasional visits from female *Osmia* bees and queen bumblebees, probably *Bombus hortorum*. *Anthophora plumipes* appear to feed mainly on *Cerinthe*, but in the grounds of the Field Station also visit *Teucrium fruticans* and *Oxalis pes-caprae* (data from analysis of pollen loads: H. Buteux, J. Bedingfield & J. Robertson, unpublished data).

General Methods

We individually marked all the *Cerinthe* inflorescences by attaching a numbered tape tag to the stem. We caught female *Anthophora* foragers at first sighting, weighed them, and uniquely marked them with coloured numbered discs (Opalithplätchen: EW Thorne, Wragby, Lincs, U.K.) or with coloured dots of quick-drying enamel paint. Bees were usually observed continuously while foraging between 0800 and 1900 hours, a period encompassing either all or the vast majority of flower visits. Flowers investigated (here called 'visited') by the bees are either accepted or rejected: for accepted flowers, bees landed on the corolla and, while hanging from it, inserted the proboscis to suck nectar, sometimes buzzing also to dislodge pollen; rejections by bees were very obvious, since the bee flew up to the flower, often touched the flower with its antennae, and flew away again after a very brief period of hovering but without landing (see Gilbert et al. 1991). Females have never been seen to collect only pollen, and uncommonly take only nectar: nectar and pollen+nectar visits were usually not differentiated in most of the data we report here.

We removed, sampled or added nectar as described previously (Gilbert et al. 1991), by inserting 1-, 5- or 10-µl microcapillary tubes to draw up the nectar by capillary action: very little detectable nectar (<0.02 µl) remains after the removal or sampling processes. We obtained volumes from measuring the height of the nectar column; concentrations were measured with a pocket refractometer (Bellingham & Stanley, Tunbridge Wells, Kent, U.K.) modified for small volumes. We added nectar by gently inserting filled microcapillary tubes into the corolla and squeezing out the solution using the delivery pipette. To measure rates of nectar renewal, we emptied 30 randomly chosen flowers of nectar, and then resampled groups of five flowers after 5, 10, 15, 30, 45 and 60 min. We assessed the amount of pollen in the anthers of accepted and rejected flowers on a scale from 0 to 5 by scoring each of the five anthers separately as 0 (no pollen), 0.5 (some pollen) or 1 (full of pollen).

For assessing rates of pollen renewal, an artificial buzzer was built from headphones whose diaphragm when in contact with the corolla caused pollen release with a buzz of the correct frequency. An appropriate buzz from a pollen-collecting female was recorded with an Aoi ECM 1035 microphone and Marantz CP430 tape recorder, digitized on computer, and rerecorded back on to a 15-s tape loop, with a 6-s gap between buzzes. The amount of pollen falling on to the speaker diaphragm was assessed on a 5-point scale (0–4); we tested 35 flowers for each of 5 consecutive min, and again 30 min later.

Statistical analysis

In much of the analysis we assessed whether several independent variables influenced the decisions made by foraging females to accept or reject flowers: the dependent variable was binary (0: reject; 1: accept), and hence logistic regression was used, implemented by GLIM (NAG Ltd, Oxford, U.K.; see Crawley 1993). The advantage of the GLIM approach is that both continuous (usually the time since last accepted) and discrete (e.g. treatment) variables can be used, and their interaction (i.e. whether the slope of the effect of time differed between groups) can be explicitly tested. The dependent variable (the decision: reject or accept) was declared as a binomially distributed variable, and hence the independent variables were fitted to the log of the odds ratio (i.e. $\ln[p/(1-p)]$, where p=the probability of acceptance: see Crawley 1993). We deleted model terms successively, starting with the highest-order interaction, retaining only those components causing significant changes in deviance upon deletion (changes in deviance are distributed as χ^2).

In the majority of the tests, we looked for the effects of time (since the previous acceptance), bee identity (usually two or three main bees in the patch), flower history (previously visited and accepted, visited and rejected, or unvisited), individual recognition (visited by the same or a different bee from the previous accepting visit) and any treatments we imposed.

All statistical tests are two tailed.

Observations

All flowers and foraging bees were individually marked, and over several days in each of 4 years, all visits to every flower were recorded (N=2577). Time gaps between revisits were then calculated, together with the identity of the bees concerned. Usually two individual females made the majority of visits, with smaller numbers from up to seven other females.

In 2 years we also recorded all rejected as well as accepted flowers (N=1006). In a logistic regression, neither year (χ^2_1=1.7, NS), day (χ^2_3=2.0, NS), time of day (χ^2_9=13.5, NS) nor any of their interactions contributed significantly to explaining the deviance in the decisions (accept/reject) made by the bees, and hence we pooled data for analysis. Using logistic regression, we tested the effect of three factors: the time since a flower was last accepted, the identity of the bee currently deciding

whether to accept or reject, and the interaction between the current and previously accepting bee (i.e. individual recognition, in two categories: same/different). We restricted the analysis to gaps of less than 60 min and the three commonest bees in each year (that made 87 and 99% of visits in the 2 years). The majority of revisits (77%) occurred after gaps of more than 3 min. We predicted that there would be a positive slope to the effect of time on the probability of acceptance, since a repellent scent mark will fade with time. Individual differences in acceptance probabilities and/or in the strength and volatility of the scent mark would generate a significant time × bee identity interaction in the model. Furthermore, we predicted that there would be a significant interaction between time and individual recognition, since an individually identifiable mark will decay with time, but a different bee should not respond, or should respond differently. Since there was a significant interaction between years (χ^2_1=10.6, P<0.001), we fitted data from each year separately.

Experiments: offering cut flowers to foraging bees

We used Williams's (1998) technique of offering cut flowers to foraging bees to test experimentally for the presence of an individual scent mark, and also to test whether bees can detect floral nectar rewards directly, without landing on the flower, independently of any scent mark. We did this by manipulating the rewards and the previous history of flowers (visited/unvisited). Picked inflorescences of *Cerinthe* placed in water survived well for 2–3 days, and were presented to foraging bees either as previously unvisited or visited (to the same or a different individual bee). Great care was taken to minimize the handling of flowers by offering them held by forceps. In all these experiments, the decision (accept/reject) and time since previous acceptance (if relevant) were recorded.

We checked whether bees could detect our handling of flowers by comparing untouched flowers (whose presentation was delayed for 30 s) with flowers probed with a blocked microcapillary (i.e. nothing added to or removed from the nectary). Across all individual bees, there was no effect of flower handling (χ^2_1=0.002, NS), nor any handling × history interaction (χ^2_1=2.5, NS). For the only female whose visits were frequent enough for analysis as an individual, while the mean proportion of acceptances of these treatments were almost identical (unmanipulated: 0.29 ± 0.08, N=31; blocked: 0.30 ± 0.09, N=27) and were not significantly different (χ^2_1=0.002, NS), there was a significant interaction with flower history (χ^2_1=7.3, P<0.01). Handling a flower increased the probability of acceptance for previously visited flowers, but decreased it for previously unvisited flowers.

Specific Experiments

Experiment 1: rewards in accepted versus rejected flowers

Naturally growing flowers were used for this set of experiments. We tested whether accepted and rejected flowers differed in nectar and/or pollen content by gently disturbing the bees as they accepted flowers, before they inserted the proboscis; we then sampled the nectar and pollen contents of these and rejected flowers (N=126 pairs over 3 years).

Experiments 2a–c: detecting floral rewards

Experiment 2a. To test whether bees could sense a sugar reward directly from naturally growing flowers, when a bee visited and accepted a flower we then alternately either added 5 µl of 30% sugar solution (N_1=58), or drained the flower of any remaining nectar and left it empty (N_2=57). We then recorded who next visited and accepted the flower, and the time it took between first and second acceptances.

Experiment 2b. We used cut flowers and real nectar to test whether bees can detect nectar directly from the smell of its trace constituents, which are absent from sucrose solutions. All flowers were unvisited before manipulation. We carried out three manipulations a total of 41 times each on previously unvisited flowers (emptying, adding 5 µl of 35% sucrose, or adding 5 µl of nectar obtained from other unvisited flowers), reusing individual flowers after more than 60 min had elapsed since the last accepting visit.

Experiment 2c. To test whether visible pollen quantities affect the probability of acceptance, we randomly selected 80 naturally growing flowers from the 272 then available, removing the anthers completely from 40 (removing both visual and olfactory cues), and colouring the pollen black in the other 40 (using a black marker pen to remove at least visual cues, perhaps at the cost of adding unwanted odour cues from the permanent ink).

Experiment 3: marking versus memory

Social bees are very good at memorizing spatial locations (Thomson et al. 1982; Thomson 1988), and therefore nonrandom foraging behaviour could result from memorizing a trapline rather than the use of a scent mark. We devised an experiment to separate knowledge of previous visits via spatial memory as opposed to a flower-specific mark such as a scent mark. We put picked similar-sized inflorescences of *Cerinthe* (i.e. single active flowers) individually into 72 small bottles filled with water, and placed these bottles in crates in an array of 12 × 6 at the edge of a large *Cerinthe* patch. Immediately after being visited (i.e. during the foraging bout of each bee), each flower was randomly assigned either to remain in its position, or to be exchanged with another in the array. This generated a set of visits to flowers and a set of visits to locations, with the two factors separated in the experimental design. Over 2 days, each visit (N_1=226, N_2=113) was categorized as an acceptance or rejection, by flower history (flower not previously accepted, or previously accepted by the same bee) and by location history (location previously unvisited, or previously visited by the same bee). Accepted flowers and visited locations were regarded as having reverted to 'unvisited' status after 60 min. There were too few visits (N=17) by bees other

than the main foraging individual for us to test for individual recognition. For previously accepted flowers and visited locations, the times since the last accepting visit were noted separately. Flower-specific characteristics such as a scent mark will move with the flowers, but spatial memory will be associated with the locations. The independent variables tested for their ability to predict the decision (acceptance/ rejection) in the logistic regression were day, time since last acceptance, location history and flower history.

Experiments 4a–d: scent marking and response to floral reward

All these experiments used Williams's technique of offering cut flowers. They were offered immediately after manipulation, and hence the first and second acceptances occurred mostly within 5 min of offering (82, 90 and 92%, respectively); these experiments were therefore concerned only with the short-term effects of scent marking.

Experiment 4a. In this experiment we tested for differences in outcome of a visit (accepted, rejected) between previously accepted (N=295) as opposed to unvisited flowers (N=36). Unvisited flowers were offered to foraging females until finally accepted, at which time they were randomly allocated either to be returned to the pool of inflorescences, or immediately reoffered to foraging bees, now as previously accepted flowers. Flowers returned to the pool remained there for at least 60 min, after which time they were regarded as 'unvisited'.

Experiment 4b. The first experiment to assess whether bees altered their marking behaviour in response to nectar volume was done over 2 successive days. Flowers (N=198) were randomly allocated to one of three treatments: control (probed with a blocked capillary); emptied (all nectar removed); and augmented (with 10 µl of 40% sucrose solution).

Experiment 4c. The second experiment with the same aim was done on a single day, and compared flowers (N=138) randomly allocated to the following treatments: control (probed with a blocked microcapillary); all nectar replaced with 1 µl of 40% sucrose solution; and all nectar replaced with 10 µl of 40% sucrose solution. In both experiments 4b and 4c, the majority of flowers had been previously visited and accepted, but a smaller subset were unvisited; those previously visited could have been visited by the same or a different bee, but the numbers of the latter group were low.

Experiment 4d. In this experiment we tested whether bees could respond to differences in the concentration of sugar solutions placed in flowers. Before presentation, flowers were randomly allocated to one of four treatments: unmanipulated but presentation delayed for 30 s; nectar replaced by 5 µl distilled water; or by 5 µl of 35% sucrose; or by 5 µl of 50% sucrose solution. Flowers were offered until finally accepted, at which time they were returned to the pool of inflorescences for at least 180 min, after which they were regarded as 'unvisited'. The timing and nature (acceptance/rejection) of the decision at every successful presentation were recorded. Bees collecting pollen were ignored in this experiment. We restricted the analysis to the three commonest females who made 97% of the visits (N=245).

Experiments 5a–b: longer-term components of the scent mark

These experiments also used Williams's technique of offering cut flowers. We looked for evidence of medium- and long-term effects of scent marking.

Experiment 5a. In this experiment, we looked for medium-term effects lasting for longer than 5 min, but less than 30 min. We allowed a marked bee to visit a flower, and then presented the flower again to either the same or a different bee, but only after a delay of 5 min (N=122). As before, the flower was presented repeatedly until either the bee finished foraging, or the flower was accepted, or 30 min had elapsed.

Experiment 5b. Long-term effects were investigated in a similar experiment, but we waited for 60 min before reoffering flowers to foraging bees (N=123).

RESULTS

Resource Renewal Rates

There was a significant double-log regression of nectar quantity against time ($F_{1,28}$=5.38, P<0.05), making the renewal process nonlinear. Nectar renewal to the previous level took 15–30 min (Fig. 1a). Flowers did not release all their pollen during one buzz (Fig. 1b): resource presentation was slightly, although not significantly, nonlinear within each set of 5 min, but there was an obvious drop in reward between each set.

Do Bees Avoid Revisiting Flowers?

From observational data, we analysed the distribution of interarrival times at flowers, recording the time between a previous and a current acceptance, only including gaps of less than 60 min (65% of observations). The distribution of such gaps was highly significantly different from the expected exponential distribution, with too many immediate revisits, and too few visits at times between 3 and 15 min (Fig. 2). This pattern is a strong indication of nonrandom foraging by the bees.

When experimentally offered as cut flowers (experiment 4a), previously unvisited flowers had a very high probability of acceptance (0.97 ± 0.03, N=36), but this was much lower (0.59 ± 0.02, N=295) for flowers that had been accepted previously within 5 min (χ^2_1=27.4, P<0.001). This contrast could also be made within two other experiments involving revisits within 5 min: in one (experiment 4b) there was no significant effect (χ^2_1=2.2,

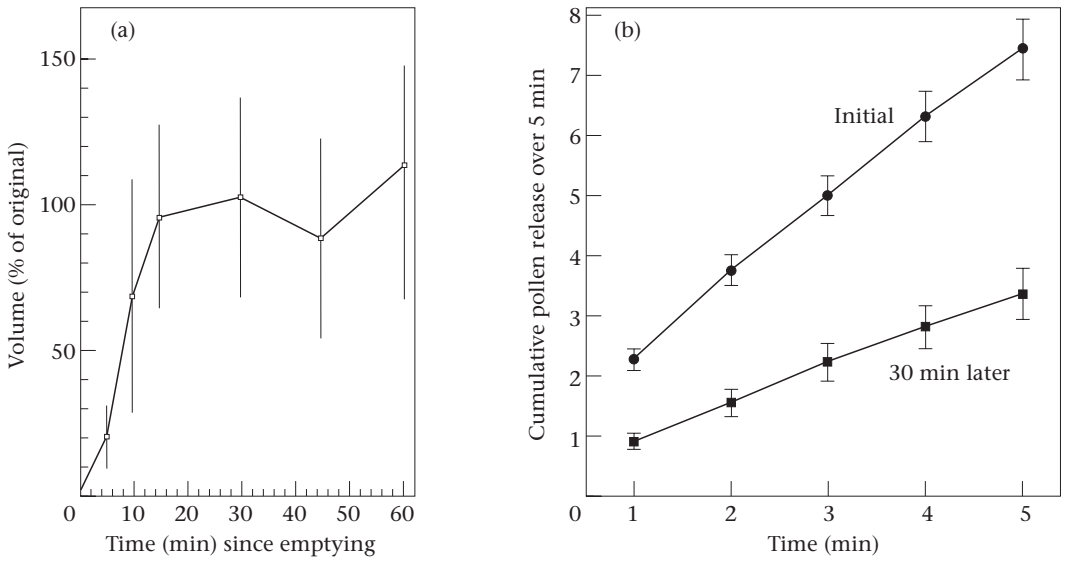

Figure 1. Resource renewal in *Cerinthe major* flowers. (a) Nectar renewal ($\bar{X}\pm$SE) as a function of time since the flower was emptied. (b) Cumulative pollen release ($\bar{X}\pm$SE) to playback of a bee buzzing; repeated every minute for 5 min, and again 30 min later. Pollen amounts released at each buzz were assessed on a 5-point ordinal scale (0–4).

NS), whereas in the other (experiment 4d) it was significant (χ^2_1=4.6, P<0.05), with previously accepted flowers having a higher probability of subsequent acceptance. Longer gaps between the first acceptance and second visit were studied in experiment 3: the probability of subsequent acceptance increased from 0.78 (previously unvisited) to 0.90 (previously accepted; χ^2_1=4.0, P<0.05). The different results of these four experiments (lower, same, higher and higher, respectively) may be caused by the increasing time between first acceptance and second visit (medians 19, 21, 42 and 1890 s, respectively).

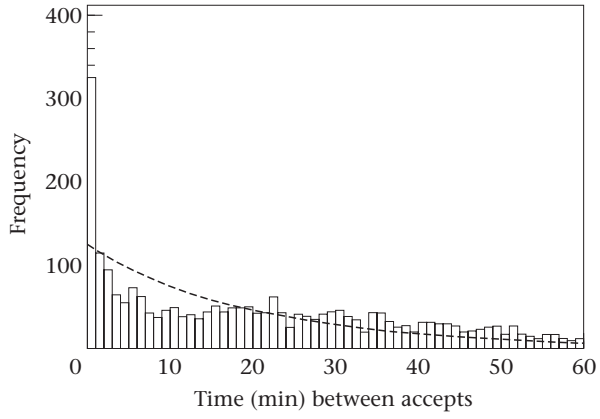

Figure 2. Distribution of interacceptance times at individual *Cerinthe major* flowers by *Anthophora plumipes* bees. The data were collected over 4 years (1994–1997); different weather conditions and bee numbers meant that there were significant differences in the median interacceptance times between years (medians of 18.2, 18.0, 24.3 and 25 min: H_3=32.4, N=2577, P<0.001). We corrected the time between accepts of different years to the same overall median of 18 min (although it makes little difference to the pattern or the statistical conclusions). The resulting distribution (shown here) is significantly different from a negative exponential distribution (the dashed line: χ^2_{59}=1141, P<0.001).

There was much more nectar (Fig. 3a, b) and pollen (Fig. 3c) in accepted flowers (experiment 1), showing a clear advantage to the rejecting behaviour shown by the bees. The pollen score and nectar content of flowers were correlated (r_s=0.30, N=80, P<0.01), and hence bees could choose more highly rewarding flowers either by sensing the nectar directly, or by selecting flowers with large amounts of pollen, either visually or by olfaction.

Is there Remote Perception of Floral Reward?

There was no effect of adding sugar solution or removing nectar from naturally growing flowers (experiment 2a) on the time to the next acceptance (H_1=0.07, NS), even when restricted to periods of less than 30 min (H_1=0.14, N_1=22, N_2=19, NS), or to cases when only the same bee revisited (H_1=0.05, N_1=N_2=16, NS). Revisits by the same bee were quicker than by a different bee (H_1=26.2, P<0.001), but this difference disappeared when considering only gaps of less than 30 min (H_1=0.36, N_{same}=25, $N_{\text{different}}$=16, NS). There is therefore no evidence from this experiment that bees sense the sugar content of nectar directly to determine whether to accept a flower.

In testing whether bees could detect real nectar as opposed to sugar solution (experiment 2b), there were differences in the overall acceptability of individual cut flowers that were used, and between bees in their overall probability of accepting flowers, but no indications that bees accepted a higher proportion of any of the treatments (emptied, added sugar solution, or added real nectar: χ^2_2=0.45, NS). Thus, there is no evidence that bees could smell trace components of real nectar before they landed on the flower.

The pollen availability (experiment 2c) manipulations (unmanipulated, or with experimentally removed or

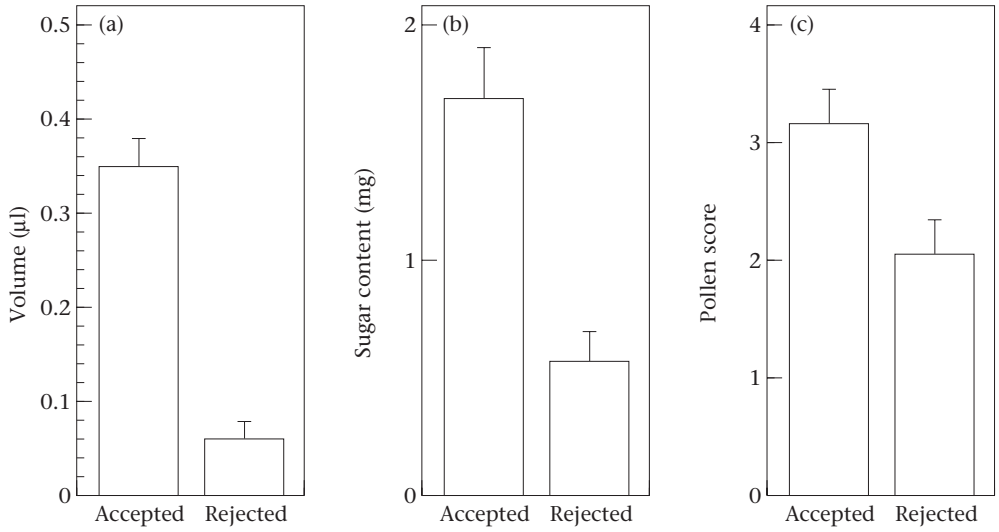

Figure 3. Comparison between the rewards (mean nectar volume, sugar content and pollen amounts+SE) of *Cerinthe* flowers accepted and rejected by foraging female *Anthophora plumipes*. Data were paired for date and time of day to allow for day-to-day and diurnal variation in nectar standing crops (N=126 pairs over 3 years). There are significant differences between accepted and rejected flowers in all three measures and also year effects on the magnitude of these differences (e.g. on nectar volume, H_2=4.1, P<0.05). (a) Nectar volume (Wilcoxon signed-ranks test: Z=5.5, N=126, P<0.001). (b) Nectar sugar content (Wilcoxon signed-ranks test: Z=4.9, N=126, P<0.001). (c) Pollen score (Wilcoxon signed-ranks test: Z=2.63, N=126, P<0.01).

blackened pollen) generated 108 visits to flowers. There was no difference in the proportion of acceptances between pollen treatments (χ^2_2=0.77, NS), nor in the time to being visited (H_2=2.15, NS) or accepted (H_2=1.48, NS). There is therefore no evidence that being able to see pollen influenced the decision to accept a flower.

Scent Marking or Memory?

Experiment 3 was designed to separate the effects of visiting the flower from those of visiting the location of the flower. The great majority of revisits in this experiment occurred after gaps of more than 3 min (median 31.5, interquartile range 13–58 min). Table 1 shows that there was an effect of the flower being visited before, but not of the location. This is unequivocal evidence for a scent mark that moves with the flower when its position is moved. The effect was in the opposite direction to the prediction, however, since a previous acceptance increased the probability of subsequent acceptance (from 0.78 to 0.90) rather than decreased it.

Testing for an effect of the time since the last acceptance also showed unequivocal support for a scent mark that travelled with the flower, rather than any decaying memory associated with the location. We restricted the analysis to visits where flowers or locations had been previously accepted by the same bee. Fitting the time since the previous acceptance to the decision at the current visit showed no effect of the time since the location was last accepted, but a clear effect of the time since the flower was last accepted (Table 1).

Effects of Time and Individual Identity

From observational data on the three main bees foraging in the patches, there were significant differences between individuals in the time until the next acceptance of a flower when either of the two most frequent visitors had been the first visitors (H_2>3.88, P<0.05). Usually, but not always, the fastest to the next acceptance was the original visitor of the flower. This may result from individual differences in experience, sensory bias, variation in arrival direction, or in flight agility; alternatively, it is consistent with some form of individual foraging route and/or scent mark.

In both years with (observational) rejection data, increasing time since the previous acceptance was highly significantly associated with an increasing probability of acceptance (χ^2_1>22.9, P<0.001). In one year (N=113), there were also significant differences between the three main foraging females in mean acceptance probability (χ^2_2=5.9, P≈0.05) and its rate of increase with time since the previous acceptance (χ^2_2=20.1, P<0.001); in addition, the predicted interaction between time and individual recognition (same/different) was significant (χ^2_1=7.1, P<0.01). We also recorded whether females were collecting pollen as well as nectar from flowers: restricting the analysis to nectar-only visits (N=62), the same patterns emerge just as strongly, with differences between bees in the rate of increase of the probability of acceptance with time (χ^2_2=19.2, P<0.001), as well as the predicted interaction between time and individual recognition (χ^2_1=6.1, P<0.01). Figure 4a plots the overall pattern, consistent with an individually recognizable scent mark; Fig. 4b shows that individuals differed in their responses to their own scent marks, whereas all responses to marks of different bees appeared to be similar.

Table 1. Results of experiment 3, in which a *Cerinthe* flower's location was changed after having been visited by a female *Anthophora*, thus separating location effects from flower scent marks (see text)

Source of variation	Change in deviance	*df*	*P*
Effect of history			
Day	4.60	1	<0.05
Location history	1.23	1	NS
Flower history	3.99	1	<0.05
Flower × location histories	0.28	1	NS
Day × location history	0.41	1	NS
Day × flower history	0.95	1	NS
Flower × location × day	1.27	1	NS
Error	265.25	265	
Final model=flower status+day			
Coefficients=log (odds ratios)			
Day 1, unvisited		0.8459±0.1905	
Addition for day 2		0.8693±0.4159	
Addition for flowers previously visited by the same bee		0.9192±0.3388	
Effect of time			
Time since location visited	0.05	1	NS
Time since flower visited	6.03	1	<0.02
Final model=time since flower visited			
Coefficients=log (odds ratios)			
	Constant	Slope	
Time since flower visited	0.97±0.37	0.0399±0.0170	

The table gives the analysis of deviance with binomial errors: effect of history, that is, unvisited as against previously visited (by the same bee) locations and flowers; and effect of the time since the last visit of the same bee. The coefficients are changes in the natural logarithm of the odds ratio (see Crawley 1993), and are tested by stepwise deletion from the full model to measure changes in deviance (here equivalent to χ^2).

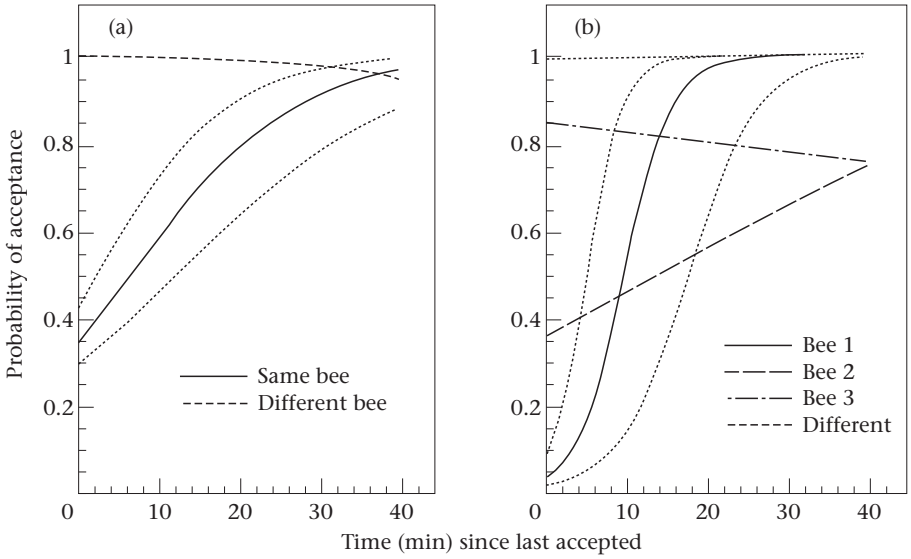

Figure 4. Results from observational data on the effect of the time since the last accepting visit on the probability of acceptance on visiting a flower of *Cerinthe* by foraging *Anthophora plumipes*. Bold lines show the changes in acceptance probability fitted via a logistic regression; dotted lines show the 95% confidence limits. (a) Flowers revisited by the same or a different bee; (b) differences between individuals in their reponses to their own marks on flowers (confidence limits shown only for the bold curve): all three responded similarly to nonself marks, and this pattern is indicated in the 'different' line.

In the other year (N=412) there were no differences between the three main foragers in mean acceptance probability (χ^2_2=1.4, NS), nor in its rate of increase with time (χ^2_2=0.6, NS), and the predicted time × individual recognition interaction was absent (χ^2_1=0.8, NS); although clearly more variable and hence not significant, the overall pattern was the same as in Fig. 4a.

Experiments Involving Revisits Within 5 min

The extremely short-term nature of one component of the scent mark was clearly demonstrated in experiment 4d, where for flowers previously accepted, we contrasted unmanipulated flowers presented immediately with those where presentation was delayed for just 30 s. There was a substantial difference in acceptance probabilities (χ^2_1=6.3, P<0.02), with delayed presentation resulting in a greatly reduced acceptability (mean 0.40) relative to immediate presentation (0.67).

In previously accepted flowers in experiment 4a, the two commonest individual bees had different overall probabilities of acceptance (χ^2_1=14.8, P<0.001), there was a strong effect of time overall (χ^2_1=13.2, P<0.001), and the predicted interaction between time and individual recognition was significant (χ^2_1=6.09, P<0.05). Immediately after being visited, an experimentally offered flower was accepted again by the same bee with a probability of 0.70 ± 0.04, but this probability surprisingly decreased with time (with a logistic slope -0.59 ± 0.17). However, if the previous visitor was a different individual, there was no decay with time, but merely a constant level of acceptance of 0.42 ± 0.12. The surprising result is that the probability of reacceptance was initially high and decreased with time in these first few minutes after an acceptance.

Experiments Involving Longer-term Revisits

In experiment 3, the majority of revisits to flowers occurred after gaps of more than 3 min (see above). As expected for a scent mark associated with the flower, the longer the time since the previous acceptance, the more likely the current decision was to accept.

In contrast, experimentally offering a flower 5 min or more after the previous visit (experiment 5a) resulted in no detectable effect of time. There was a strong effect of individual recognition (χ^2_1=10.5, P<0.01), but none of time (χ^2_1=0.04, NS), nor a time × individual recognition interaction (χ^2_1=1.5, NS). One female made the majority of the visits, and was designated the 'major', with others called 'minors': there was an interaction in the responses of these two categories (χ^2_1=3.9, P<0.05: see Fig. 5) that showed that the major female accepted (and presumably marked) flowers previously marked by either herself or by others, whereas minors avoided visiting flowers marked by the major, but not those marked by other minors.

The results of waiting for 60 min instead of only 5 min (experiment 5b) were similar to those of the previous experiment: there was no effect of time (χ^2_1=0.01, NS), but there was a strong effect of individual recognition (Fig. 6: χ^2_1=13.0, P<0.001). By this time the major bee had disappeared and so we were unable to repeat the previous analysis. However, separating the original marking and subsequent deciding bees into the individuals concerned (rather than the same versus different categorization) showed that there was a strong interaction among these individuals (χ^2_{20}=41.2, P<0.01): in her decision whether to accept a given marked flower, it clearly mattered to a female exactly who had previously accepted it.

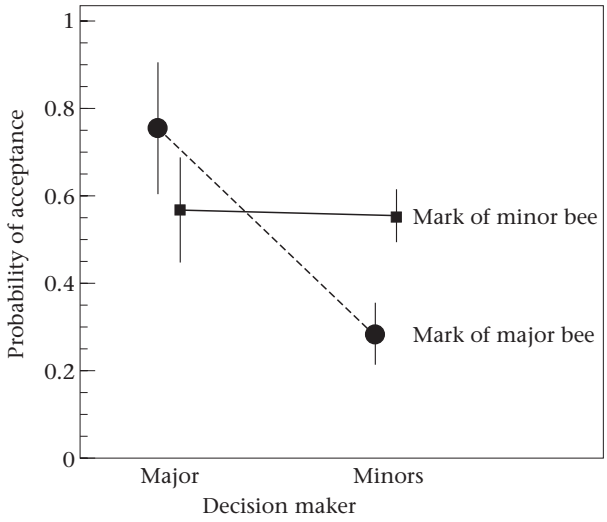

Figure 5. Responses of the female *Anthophora* that made the majority of visits ('major') and the others ('minors') to each other's marked flowers. Cut flowers were allowed to be visited once by either the major or any of the minors, and then presented again to foragers after 5 min (to remove the effects of the short-term mark). Lines connecting the mean values for majors and for minors are for ease of interpretation only, and do not imply continuity.

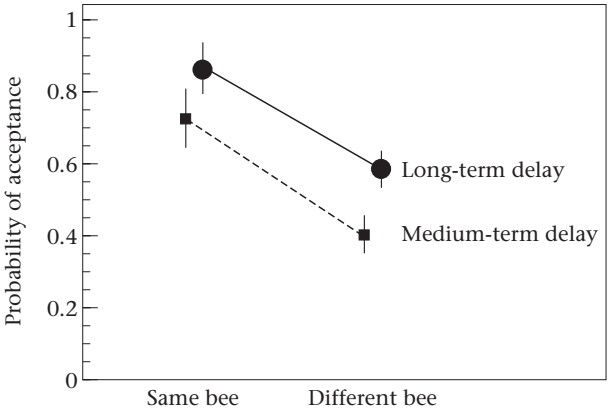

Figure 6. Responses of female *Anthophora plumipes* to their own scent mark, or to that of a different female, and how this changed with time. Visited flowers were kept unvisited for between 5 and 30 min ('medium-term delay') or more than 60 min ('long-term delay'). Lines connecting the mean values for long-term and medium-term delays are for ease of interpretation only, and do not imply continuity.

Putting the data for experiments 5a and 5b together (see Fig. 6), there was a significant increase in the overall probability of acceptance between medium and long delays (χ^2_1=8.5, P<0.01), a huge effect of individual recognition (χ^2_1=19.1, P<0.001), but no interaction (χ^2_1=0.02, NS).

Is Marking Altered by Reward Level?

In experiment 4b (comparing control, emptied and augmented, 10-µl, flowers), there was no effect of previous bee identity (same/different, χ^2_1=0.01, NS, although

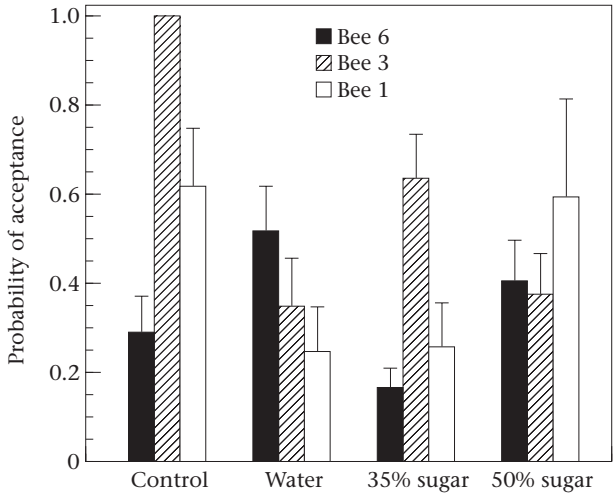

Figure 7. The probability ($\bar{X}$+SE) of accepting an experimentally offered *Cerinthe* flower in relation to nectar concentration treatment and bee identity for three female *Anthophora* bees.

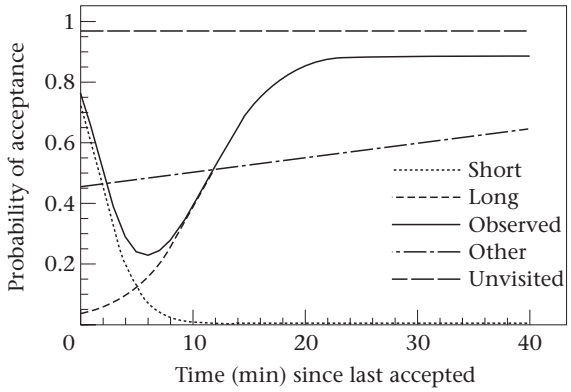

Figure 8. Summary of our interpretation of the way in which scent marks are used by female *Anthophora* bees foraging on *Cerinthe major* flowers in Portugal. The scent mark of a bee revisiting its own marked flower has two components: a very short-term component ('short') and a longer-term component ('long'), which combine to give the observed pattern of acceptance probability ('observed'). The possibility of even longer-term components is indicated by the higher overall level of the 'observed' line over that of flowers marked by a different bee ('other'). Flowers marked by a different bee gradually become more acceptable with time, until they effectively become 'unvisited' flowers.

sample sizes for 'different' bees were small), but individual bees accepted flowers with differing probabilities (χ^2_4=11.7, P<0.02). Because of the strong day effect (χ^2_1=10.6, P<0.01), we then analysed each day separately. On one day (N=54), there was a significant difference in the effect of the time between treatments (χ^2_2=6.2, P<0.05), with control and emptied flowers showing no effect of time, but augmented flowers having a significant negative slope (-0.43 ± 0.29). On the other day (N=144), there were no differences in slopes between treatments (χ^2_2=2.1, NS).

When comparing control, 1-µl and 10-µl volumes of sugar solution in flowers (experiment 4c), we had few unvisited flowers, or flowers revisited by different bees, and hence analysis concentrated upon revisits to flowers previously visited by the same bees (N=138). There were differences between treatments in the slope of the effect of time on the probability of acceptance (χ^2_2=12.0, P<0.01), with the slope being significantly negative for flowers augmented by 10 µl of sugar solution, but zero for both the other treatments. Individual bees again responded with differing patterns of acceptance probabilities to treatments (χ^2_6=13.2, P<0.05).

When comparing marking behaviour in response to different concentrations of added sucrose solution (experiment 4d), the major component was the substantial differences in responses to the treatments by individual bees (Fig. 7: χ^2_6=25.4, P<0.001). One bee had a very reduced probability of acceptance for 35% sucrose over either water or 50% sucrose, whereas another had a reduced response to the water treatment. For flowers previously accepted (N=173), we looked for the predicted interaction between the time since previous acceptance and individual recognition, as well as for the effect of the treatment manipulations. The final model contained only three significant components: as before, there were differences in the responses of different bees to the treatments (χ^2_6=38.9, P<0.001) and also in the slopes of the effect of time on these responses (χ^2_5=13.0, P<0.05). Furthermore, the time × individual recognition interaction was present (χ^2_1=9.9, P<0.01). The coefficients show that if the previous visitor had been a different individual, the probability of acceptance was low with no effect of time, whereas for the same bee revisiting the flower the acceptance probability started much higher and became higher still with time (logistic slope 0.11 ± 0.05).

DISCUSSION

Our results indicate that female *A. plumipes* used scent marking in their foraging behaviour, that these marks were individually recognizable, and that individual females varied in the way in which they mark in response to reward levels, and possibly also in response to their own physiological state, competitive ability or status. They probably used at least two different scent components to help them exploit arrays of flowers of *C. major* in Portugal (Fig. 8). A very short-term component lasting only 1–3 min was attractive, producing a substantial increase in the probability of revisiting and probing a flower. Then a longer-term repellent scent component lasting for 20–30 min made rejection of a flower more likely. Marked flowers were treated differently over much longer periods too, raising the possibility of further components of even less volatility. Such components, if they exist, increased the probability of acceptance over unvisited flowers. This last interpretation is uncertain, however, since in some years and some circumstances unvisited flowers were more attractive (cf. Fig. 4a, b) or less attractive than previously visited flowers. We think this outcome depends on the length of time the experiment allows between the previous and current visit: the

longer this time is, the more attractive already marked flowers are relative to unmarked ones (see Results).

The first, individually based, very short-term mark is the most puzzling. It was clearly shown in some of the manipulations (experiments 4a, b, c) involving short revisit times, but only when a female revisited the same flower she herself had just accepted. There was no indication that a female responded to another female's mark, since all the relevant slopes with time were not significantly different from zero. Bees also appeared to use this mark only when reward levels were high, since such marks were evident only on flowers with augmented rewards (experiments 4b, c). Why should a bee mark a flower so that it can immediately revisit it? In fact, when watching females foraging among *Cerinthe* flowers, such immediate revisits were a noticeable feature (Fig. 2). It could be that females were unable to remove all the nectar or pollen in one go, and required at least a second visit. Not all available pollen was released during a single visit (Fig. 1b), possibly making it profitable to revisit immediately: a similar behaviour was seen in *Anthophora pauperata* Walker foraging on another Boraginaceae plant (*Alkanna orientalis* (L.) Boiss.) in Sinai (Willmer et al. 1993; Stone et al. 1999). The amounts of nectar left behind by bumblebees and honeybees is typically very small, less than 0.02 µl, so small that an immediate revisit would be unprofitable (Williams 1998). We have found that *Anthophora* females typically leave small amounts of nectar (5 µg sugar, N=12) after an acceptance visit, but immediate revisits were to flowers with much more nectar left in them (75 µg sugar, N=5). The data are too scanty to make strong conclusions, but to judge from Williams's (1998) model, females could more than cover their costs by revisiting immediately. There may be physical, mechanical or physiological reasons why nectar was left behind after a visit, but at the moment we have little idea of the true cause. An alternative idea is that this short-term mark helps in area-restricted searching, enabling them in the short term to remain in profitable patches of flowers, but it does not seem to last long enough for this to be effective.

The second, repellent component has been studied to a certain extent in bumblebees (Cameron 1981; Schmitt & Bertsch 1990; Goulson et al. 1998; Stout et al. 1998; Williams 1998) and honeybees (Williams 1998). In these taxa, this scent mark seems not to be individual- or species-specific, but rather is a generalized mark to which other species, or at least congenerics, also respond (Stout et al. 1998; Williams 1998). There was good evidence for this kind of scent mark in *Anthophora* too (observational data, experiment 2; perhaps experiment 4d), and the data suggest it usually lasted about 20–30 min. We have found the typical duration of this mark to be variable between years; the data (experiments 4b, c, d) strongly suggest that the strength of this scent component is varied by the bees in response to their perception of the reward, and hence probably to their estimate of the rate of secretion.

Figure 4b is intriguing. The individual bee making most of the flower visits in the patch, the 'major' bee, produced a mark to which its own response faded within 20 min; the other two bees responded to their own scent marks quite differently; and all three bees visited and accepted any flowers that had been marked by a different bee. This pattern is reinforced by the results of experiment 5a, which showed (Fig. 5) that minor females avoided visiting flowers marked by the major, but the major did not avoid flowers marked by minors. This may be because the marks made by minors evaporated more quickly than those of the major bee, but not because of the frequency of marking (because this was controlled experimentally); alternatively the major bee may be countermarking the marks of minors instead. It is therefore tempting to speculate that these marks are involved in the creation and maintenance of a dominance hierarchy or some other exclusivity mechanism among females. Countermarking in mammals using urine (Gray & Hurst 1997) and countersinging by overlapping a rival's song in birds (Dabelsteen & McGregor 1996) are considered to be particularly aggressive responses by a dominant territory holder to signals from intruding individuals. Scent marking of flowers may play a similar role in social communication among *Anthophora* bees. Overt and obvious aggression among females is a regular feature of foraging *A. pauperata* in Sinai (Willmer et al. 1994; Gilbert 1999).

Over the longer term of hours and days, the effects of marking flowers are still detectable, since there is an attractive component evident in experiments 2 and 5b. It is hard to tell whether this is a different sort of low-volatile component, or the end result of the fading of the repellent mark. Marked flowers became more attractive with time, even times >1 h (see Fig. 6). This unanticipated result was also disconcerting, since we had regarded flowers left for 60 min as effectively 'unvisited' in many of our experiments prior to this discovery. Such longer-term effects probably serve to identify a foraging route or area for individual females, renewable from day to day, or perhaps lasting overnight or over several days, as probably occurs in bumblebees. Individual females do return every day to the same patch of flowers, and different females regularly forage in predictable but different areas of the same array of flowers, but there is no sign of any true traplining behaviour as is obvious in bumblebees (F. Gilbert, unpublished data and traplining statistical test). It is certainly possible that a female uses the repellent mark to avoid revisiting flowers too soon after emptying them, but when it has faded enough to indicate that more secretion has probably occurred, she may use it as an aide mémoire to help her find the flower again: the response threshold at which this dose-dependent reversal of response occurs may be individually variable and set by her own experience, and probably also depends upon the replenishment rate of the reward in the flowers (S. A. Corbet, personal communication).

An alternative scenario is equally plausible (S. A. Corbet, personal communication). The two components of the scent mark posited here may be different marks altogether, placed on flowers in different circumstances by different bees. The short-term 'bookmark' is used only if a forager cannot complete the removal of the reward from a flower, and it helps her to complete the visit; pollen-collecting honeybees and bumblebees often interrupt their visits to hover and pack their booty into their

pollen sacs before resuming collection. The other type of mark may be made only by a trapliner or a territory owner, and is placed on a flower that has been visited and emptied. It reminds her not to visit that flower for a period of time (adjusted to her perception of the renewal rate), but when sufficiently faded encourages her to revisit. Other bees may learn to avoid this mark because they may not have sufficient knowledge of the local flowers to ascertain what it means. Whereas the trapliner/owner's foraging path is largely a response to a systematic network of her own scent marks, that of a nontrapliner/nonowner is more probably a matter of opportunistic guesswork in which scent marks figure less prominently.

Traplining may form a worthwhile foraging strategy that will deter intruders even if flowers have identical secretion characteristics (Possingham 1989), but there are often large differences in nectar secretion rates between plant individuals that may further enhance the strategy. We have shown that flowers and plants of *Cerinthe* differ substantially in their rates of nectar production, consistent with an ESS model of plant–pollinator coevolution (Gilbert et al. 1991, unpublished data). A long-lasting effect of scent marking may therefore also serve to identify these particularly rewarding flowers. Whether female *Anthophora* use scent marks to discriminate between rewarding and unrewarding flowers will be important in the context of a coevolutionary interpretation of this plant–pollinator relationship.

In agreement with the suggestions for Possingham's (1989) model, we think that the evidence points to the longer-lasting individually recognizable scent mark being one mechanism by which females may compete with one another for floral resources, perhaps via the mechanism of the systematic exploitation of a flower array, enabling a profit to be made by a knowledgeable forager where a naïve forager would fail (Corbet et al. 1984; Possingham 1989). Bumblebees denied the opportunity to use such attractive marks had a reduced rate of energy intake (Schmitt & Bertsch 1990). How this interacts with the possibility of a dominance hierarchy is not known, but clearly needs further research.

The use of scent mark components of differing volatility represents a complex and sophisticated method of exploiting a highly rewarding plant, easily equal to the techniques demonstrated in bumblebees. We have shown that individual females responded very differently to particular floral treatments. Individuals placed marks that differed in their volatility, at least sometimes partly in response to perceived reward levels. The resource needs and competitive status of individual females probably contributed to these different responses. Such extraordinary flexibility entails a new appreciation of the ways in which solitary bees respond to their foraging environment.

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