Bumble-bee learning selects for both early and long flowering in food-deceptive plants

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Most rewardless orchids engage in generalized food-deception, exhibiting floral traits typical of rewarding species and exploiting the instinctive foraging of pollinators. Generalized food-deceptive (GFD) orchids compete poorly with rewarding species for pollinator services, which may be overcome by flowering early in the growing season when relatively more pollinators are naive and fewer competing plant species are flowering, and/or flowering for extended periods to enhance the chance of pollinator visits. We tested these hypotheses by manipulating flowering time and duration in a natural population of Calypso bulbosa and quantifying pollinator visitation based on pollen removal. Both early and long flowering increased bumble-bee visitation compared with late and brief flowering, respectively. To identify the cause of reduced visitation during late flowering, we tested whether negative experience with C. bulbosa (avoidance learning) and positive experience with a rewarding species, Arctostaphylos uva-ursi, (associative learning) by captive bumble-bees could reduce C. bulbosa’s competitiveness. Avoidance learning explained the higher visitation of early- compared with late-flowering C. bulbosa. The resulting pollinator-mediated selection for early flowering may commonly affect GFD orchids, explaining their tendency to flower earlier than rewarding orchids. For dissimilar deceptive and rewarding sympatric species, associative learning may additionally favour early flowering by GFD species.

Keywords: associative learning; avoidance learning; deceit pollination; flowering phenology; orchid

1. INTRODUCTION

Deceit pollination, whereby plants offer neither nectar nor harvestable pollen to their pollinators and instead rely on their pollinators’ expectations of food or mates, bears obvious risks of limited visitation and pollination failure. Deceptive species compete poorly for pollinator services with rewarding species [1] because they provide no resources, which probably reduces their continued attraction of individual pollinators. Pollinator learning accentuates this limited competitiveness in two ways. First, through reinforcement by repeated ingestion of nectar and/or pollen, pollinators of rewarding species can learn positive associations between floral signals and rewards that enhance these species’ attractiveness relative to that of deceptive species (associative learning) [2]. Second, the lack of reward during visits to deceptive species can promote pollinator learning of adverse associations of floral traits with lack of success, increasing pollinator avoidance of such species (avoidance learning) [3]. Learning has limited effects on deceptive species that specifically mimic flowers of co-occurring rewarding species [4,5], because satisfactory pollinator experience with the models provides vicarious reinforcement that motivates continued visitation to mimics. In contrast, learning bears more severe consequences for species engaged in generalized food-deception, which exploit the instinctive expectations of opportunistic pollinators [6] for general floral signals (colour, fragrance and inflorescence shape) [7], but cannot rely on rewarding species to reinforce pollinator associations. Such deceitful species are often pollinated by either inexperienced flower visitors or experienced visitors whose preferred food source has been depleted [7,8], and they commonly experience low visitation and pollination success [5].

Despite the problems associated with generalized food-deception, it is the most common pollination mode [7] among the third of orchid species that rely on deceit pollination for outcrossing [9,10]. The frequent and often severe pollen limitation that they typically experience [11] should select strongly for reproductive traits that enhance pollination [12], including flowering schedules that promote pollinator visits and pollen exchange among conspecifics [13]. Two flowering patterns may allow generalized food-deceptive (GFD) orchids to minimize the detrimental effects of competition for pollinators with rewarding species and pollinator learning. First, long-lived flowers may enhance opportunities for pollination [14,15] by both enhancing the probability and number of pollinator visits [16] and decreasing flowering overlap with co-occurring species, thereby reducing competition for access to pollinators. Second, in seasonal environments, flowering early may directly promote pollination of GFD orchids [13]. Strong competition selects for staggered flowering phenologies of co-occurring plant species [17–19]. As deceptive species are typically more pollinator limited than rewarding species [5], their flowering time should be most responsive to such selection. In temperate areas, flowering during early spring may allow GFD orchids to avoid the flowering peaks of most co-occurring rewarding species and thus to experience lower competition for pollinators [13]. Additionally, the proportion of inexperienced pollinators should generally be higher early during the flowering season than later. All deceitful orchids are pollinated by insects with annual life cycles, so that in each flowering

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season all potential pollinators have a naive period before they learn which plant species are rewarding [1,20,21]. Flowering early when most pollinators are naive should expose GFD orchids to more frequent visits, even though more individuals of pollinating species are active later. This advantage would be accentuated when late-emerging insects learn faster than early-emerging ones, because the former learn from the latter [22].

We assessed several general hypotheses concerning floral longevity and flowering time in GFD orchids, using *Calypso bulbosa* (L.) Oakes var. *americana* (R.Br.) as an experimental model. This GFD orchid flowers in spring and is pollinated primarily by queen *Bombus* and *Psithyrus* bumble-bees [23,24], before the emergence of worker bees. Each reproductive *C. bulbosa* ramet produces a 5–20 cm scape with a solitary flower that provides pollinators neither nectar nor usable pollen, but instead relies on bright colours, anther-like hairs and a sweet smell to deceive pollinators. To test whether flowering early for a protracted period enhances pollinator visitation and reproductive success, we manipulated flowering time (early versus late) and duration (brief versus long) in a natural population. To determine how flowering time affects pollination of deceptive orchids, we additionally assessed the relative influences of associative learning through positive experience with rewarding *Arctostaphylos uva-ursi* (L.) Spreng. (Ericaceae), and avoidance learning during previous experience with *C. bulbosa* on subsequent pollinator visits to *C. bulbosa*.

2. MATERIAL AND METHODS

(a) Study site

We used populations of *C. bulbosa* and *A. uva-ursi* adjacent to the University of Calgary’s Barrier Lake Field Station in the Front Range of the Rocky Mountains, Alberta, Canada (115°03′N, 51°01′W). In this area, both species are among the first to flower in spring and are pollinated by the same bee species. *Arctostaphylos uva-ursi* occurs along the edges of mossy, coniferous forests occupied by *C. bulbosa* [23]. During 2009, the *C. bulbosa* study population included 774 ramets, of which 281 produced a flowering stalk. Flowers opened continuously between mid and late May, and average flower lifespan was 10.2 ± 2.05 days, so that some flowers were exposed from mid-May to early June, with a peak between 20 and 25 May. Nearby, *A. uva-ursi* flowered from mid-May to mid-June, starting slightly later than *C. bulbosa*. Bumble-bee queens were first observed in mid-May, roughly coincident with the beginning of *C. bulbosa* flowering.

(b) Flowering phenology and duration

During May and June 2009, we investigated the effects of two fully crossed factors, flowering time (early versus late) and flowering duration (brief versus long), on pollen removal from *C. bulbosa*. A *C. bulbosa* flower produces a single, solid pollinarium, which must be removed by a pollinator; therefore, the absence of a flower’s pollinarium demonstrates that it had been visited at least once. We selected 19 patches containing at least four reproductive ramets. Before anthesis, we enclosed each flower in a mesh bag to prevent pollinator visits. Within each patch, we randomly selected four flowering individuals (i.e. 76 ramets total) and randomly assigned them to one of four treatment combinations that differed in exposure to pollinators during the 9-day experimental period (21–29 May, i.e. during peak flowering):

- early and brief flowering: days 1–3;
- early and long flowering: days 1–6;
- late and long flowering: days 3–9; and
- late and brief flowering: days 6–9.

This design ensured that two flowering ramets in each patch were exposed simultaneously to pollinators during the 9 days, hence avoiding potential confounding effects of flower abundance, while allowing availability of fresh flowers within each patch during the experimental period.

We measured plant height and labellum length and width for each plant during the day prior to its exposure to pollinators and recorded flower age (days since anthesis) on the exposure day. Most of these variables did not vary significantly with exposure time or duration or their interaction (p > 0.15; two-factor, randomized block ANOVA). The exceptions involved labellum length, which was greater for flowers exposed late (mean ± s.e. = 1.96 ± 0.02 cm) than for those exposed early (1.88 ± 0.02 cm; F1,54 = 13.55, p < 0.001) and flower age at exposure, which was subject to interacting effects of exposure time and duration (F1,54 = 8.72, p < 0.005). In particular, flowers exposed early averaged 2.7 (±0.25) days of age, regardless of exposure duration (F1,54 = 0.76, p > 0.3), whereas among flowers exposed late those exposed for 3 days were initially older on average (5.0 ± 0.3 days) than those exposed for 6 days (3.5 ± 0.3 days; F1,54 = 25.46, p < 0.001).

Starting on 20 May (day 0), plants were unbagged between 22.00 and 24.00 h, thus ensuring that all plants assigned to an early-flowering treatment were exposed simultaneously to pollinators. Because tree cover can affect pollen removal and the risk of frost damage in *C. bulbosa* [24], we estimated the percentage tree cover above each patch each day from photographs taken between 09.00 and 12.00 h with a fisheye lens (Nikon DX AF 10.5 mm, 1 : 2.8 G ED). For each picture, we measured the percentage canopy cover based on the percentage of black pixels (number of black pixels/total number of pixels) in a high-contrast version of the image. We estimated bumble-bee abundance by counting the bees that foraged at the study site during 3 h each day. Average daily bumble-bee abundance did not vary among the four treatment combinations (permutation tests on mean squares; treatment: m.s. = 0.315, d.f. = 3, p = 0.993; residuals: m.s. = 10.345, d.f. = 14). At the end of each exposure period, we bagged each flower between 22.00 and 24.00 h and recorded pollinarium removal the following day. On 6 June, a snow storm damaged most flowering stems in our study population, precluding measurement of fruit and seed set. Nevertheless, the aggregation of pollen into solid pollinia in many orchids, including *C. bulbosa*, greatly reduces pollen losses during removal and transport [25], enhancing the reliability of pollen removal as a measure of successful pollen transfer. In particular, a previous study of the population we examined found that 39.7 per cent of the pollinia removed from flowers were subsequently detected on conspecific stigmas [26].

We used a generalized linear mixed model to assess the effects on pollinarium removal of flowering time, flowering duration (crossed, fixed factors), plant height, labellum length, labellum width, percentage of tree cover and flower age (fixed covariates), and patch (random factor). This analysis employed the glm.mQ function in R v. 2.11.1 (R Foundation for Statistical Computing, Vienna, Austria) and considered a binomial distribution with a logit link function.
(c) Pollinator learning
During May and June 2010, we used worker Bombus impatiens (Cresson) with no prior experience visiting flowers of any type from two captive colonies to assess the effects of associative and avoidance learning on visitation to C. bulbosa. Each bumble-bee was exposed individually to one or two of the following treatments:

- **Naïve**. Naïve bees exposed to 10 C. bulbosa flowers;
- **Associative learning**. Naïve bees first visited 10 A. uva-ursi inflorescences and then encountered 10 C. bulbosa flowers during their next foraging bout; and
- **Avoidance learning**. Bees initially subjected to the Naïve treatment encountered a second set of 10 C. bulbosa flowers.

These treatments were conducted in a laboratory near the field site, providing ready access to fresh flowers. Fresh flowering stems of C. bulbosa (previously enclosed in a mesh bag to prevent pollinator visits) or A. uva-ursi inflorescences bearing 4–5 flowers in individual water-filled vials were placed in a flight cage (1.83 m wide × 0.76 m deep × 0.91 m tall) illuminated by three full-spectrum fluorescent bulbs (General Electric, F40PL/AQ) and one blacklight fluorescent tube (Standard, F40T12/BL/RS) to simulate natural sunlight. A bee was then released into the cage from its attached colony and allowed to visit flowers until she returned to the hive. We recorded each trial with a digital video camera (Panasonic, SDR-H40PC) to quantify bee behaviour. From these recordings, we counted the approaches within 2 cm of C. bulbosa flowers, landings, proboscis extensions and probes for each bee. After trials with C. bulbosa, we counted the flowers with pollinia removed or deposited. Bees remove and/or deposit pollinia only when they probe C. bulbosa flowers in the appropriate position [27].

We used generalized linear models to test the effects of treatment (associative learning, avoidance learning and naïve) and colony on the numbers of approaches, landings, proboscis extensions, probes, pollinia removed and pollinia deposited. Analysis of pollinaria removal and deposition considered bees from only one colony, because no bees from the second colony removed or deposited pollinia (presumably because of their relatively small size). Because individual bees experienced two treatments, we used generalized estimating equations [28] to account for the effects of correlated responses and score statistics ($T$) to test null hypotheses. These analyses considered a compound-symmetric variance–covariance matrix. We also tested whether the frequency of a behaviour depended on the frequency of the preceding behaviour in the approach–land–extend proboscis–probe sequence, by including the number of observations of the preceding behaviour as a covariate. For analyses of pollinaria removal and deposition we used two models that either included or not the number of flowers bees probed during individual trials. These analyses were conducted with the GENMOD procedure of SAS v. 9.2 (SAS Institute, Cary, NC, USA) with a negative binomial distribution and a ln-link function. We used the Dunn–Sidák procedure to control the experiment-wise type I error rate to $a = 0.05$ [29] for multiple comparisons between least-square means for different treatments.

Our use of worker, rather than queen, bees of a species that does not naturally pollinate C. bulbosa in a flight cage should detract little from the ability of our laboratory experiment to test general features of floral deceit for several reasons. *Calypso bulbosa* is pollinated by diverse bumble-bee species, both locally and throughout its Holarctic range [23,24], and so is likely adapted for general interactions with bumble-bees, regardless of species. Furthermore, although bumble-bee cognition and learning have received little comparative analysis, Raine et al. [30] found similar colour preferences for eight Bombus species from Europe, Asia and North America, and Laverty [31] found no difference in learning rate among three generalist Bombus species, including B. impatiens. Whether queens and workers learn similarly remains unexamined, presumably because queens are uncooperative subjects, being generally less motivated foragers than workers. The only obvious consequence of our use of workers, which must be considered in interpreting the results, is their smaller bodies, which reduces the chance that a visiting bee will contact the sexual column and remove and/or deposit pollen. Finally, that this experiment was conducted indoors in a flight cage likely had little effect on the incidence of associative and/or avoidance learning. Our subjects had no prior foraging experience that could shape their expectations and the natural light spectrum allowed them to perceive floral signals normally. Therefore, the experimental conditions seem appropriate for testing mechanistic hypotheses about the role of learning in floral deceit.

3. RESULTS
Pollinaria removal from C. bulbosa flowers (and hence pollinator visitation) in the field experiment varied significantly with only flowering time and duration, and labellum length. Both long flowering ($t_{49} = 4.19, p < 0.001$) and early flowering ($t_{49} = 2.86, p < 0.01$) elevated pollinaria removal compared with brief and late flowering, respectively (figure 1). These effects acted independently, as indicated by the non-significant interaction between flowering time and duration ($t_{49} = 0.002, p > 0.99$). Flowers with long labella were more likely to experience pollinaria removal ($t_{49} = 2.56, p < 0.05$, partial regression coefficient ± s.e. = $6.52 ± 2.56$), indicating a role in either pollinator attraction to C. bulbosa or the mechanical fit between bumble-bees and C. bulbosa flowers necessary for pollination. None of the other covariates, namely flower age ($t_{49} = 1.55, p > 0.1$), labellum width...
Table 1. Results of generalized linear models assessing the effects of treatment (naïve, prior experience with Calypso bulbosa, prior experience with Arctostaphylos uva-ursi), bee colony and the frequency of the preceding behaviour in the approach-landing-proboscis extension-probing sequence on bee behaviour and pollination during visits to Calypso flowers. All analyses considered ln-link functions. Significance tests involved score statistics ($T$).

<table>
<thead>
<tr>
<th>dependent variable</th>
<th>treatment</th>
<th>colony</th>
<th>preceding behaviour</th>
<th>partial regression coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>approaches landings</td>
<td>$T_2 = 23.11^{***}$</td>
<td>$T_1 = 4.48^*$</td>
<td>$T_1 = 11.66^{***}$</td>
<td>$0.031 \pm 0.005$</td>
</tr>
<tr>
<td>extensions</td>
<td>$T_2 = 18.68^{***}$</td>
<td>$T_1 = 8.63^{***}$</td>
<td>$T_1 = 14.71^{***}$</td>
<td>$0.102 \pm 0.013$</td>
</tr>
<tr>
<td>probes</td>
<td>$T_2 = 23.31^{***}$</td>
<td>$T_1 = 10.16^{**}$</td>
<td>$T_1 = 12.90^{***}$</td>
<td>$0.178 \pm 0.028$</td>
</tr>
<tr>
<td>pollinarium removals</td>
<td>$T_2 = 12.36^{**}$</td>
<td>$T_1 = 11.04^{***}$</td>
<td>$T_1 = 9.22^{**}$</td>
<td></td>
</tr>
<tr>
<td>pollinarium depositions</td>
<td>$T_2 = 16.66^{***}$</td>
<td>$T_1 = 9.22^{**}$</td>
<td>$T_1 = 4.48^*$</td>
<td>$0.235 \pm 0.055$</td>
</tr>
<tr>
<td></td>
<td>$T_2 = 6.74^{*}$</td>
<td>$T_1 = 4.48^*$</td>
<td></td>
<td>$0.235 \pm 0.055$</td>
</tr>
</tbody>
</table>

$t_{49} = 0.11, p > 0.9$, plant height ($t_{49} = 0.61, p > 0.5$) and per cent tree cover ($t_{49} = 1.54, p > 0.1$), significantly affected pollinarium removal.

The numbers of approaches, landings, proboscis extensions and probes by bumble-bees varied significantly among treatments in the laboratory experiment (table 1). Overall, prior experience visiting A. uva-ursi increased the frequencies of responses during subsequent visits to C. bulbosa flowers compared with approaches and landings by naïve bees (table 1). However, inclusion of the preceding behaviour in the visit sequence eliminated the beneficial effect of prior experience with a rewarding species on the frequencies of proboscis extensions and probes (table 1 and figure 2), but not landings. These results are consistent with associative learning, but indicate that it primarily affects initial pollinator attraction, rather than the post-attraction responses necessary for pollen collection. Prior experience with C. bulbosa flowers inhibited all behaviours during subsequent exposure to C. bulbosa flowers (figure 2), regardless of whether the analyses considered the frequencies of the preceding behaviour in the visit sequence (table 1). These results are consistent with avoidance learning and indicate that it affects all components of the visit sequence. Bees from the first colony approached, landed, extended their proboscis and probed more C. bulbosa flowers than those from the second colony (table 1), suggesting genetically determined variation in the propensity of bees to exhibit these behaviours.

Effects of learning on pollinator behaviour correspondingly affected pollination. Prior experience with C. bulbosa reduced pollinarium removal during subsequent exposure to C. bulbosa flowers relative to their initial exposure (figure 2). However, this effect became non-significant when we accounted for the number of C. bulbosa flowers probed, indicating that the reduction in pollinarium removal is due to bumble-bee behaviour. Finally, pollinarium deposition did not differ among treatments (table 1 and figure 2).

4. DISCUSSION
(a) Do early and long flowering enhance pollinator visitation to generalized food-deceptive orchids?
Early and long flowering independently enhanced natural pollinator visitation to C. bulbosa when compared with late and brief flowering, indicating that selection favours early anthesis of long-lived flowers in C. bulbosa, all else being equal, because it maximizes opportunities for pollination and mating [14]. This conclusion is consistent with the observation that pollinator limitation was the main constraint on fruit set in a Swedish population of C. bulbosa during three consecutive years [24]. Genetic responses to such selection on phenological traits would be accentuated by the pleiotropic control of anthesis timing and flower longevity in orchids [15,32]. However, factors such as weather, herbivores and/or disease can oppose pollinator-mediated selection on flowering phenology [33]. Most obviously for the population we studied, spring frost may strongly counteract selection for early flowering. Frost caused almost complete fruiting failure in two of the five years that this population has been studied (26), this study and earlier studies by H. C. Proctor & L. D. Harder 2011, personal communication) and this frequency would increase if flowering occurred even earlier. The outcome of such opposing influences on selection will depend on their relative frequencies during the lives of individuals.

Pollinator-mediated selection for early flowering may occur commonly in many GFD orchids. For example, beneficial effects of early flowering on visitation or reproductive success have been observed in Anacamptis morio [34], Changnienia amoena [35], Cypripedium japonicum [36], Dactylorhiza sambucina [6] and Orchis mascula [37]. More generally, the recent finding that GFD orchids start flowering earlier than rewarding orchids [38] indicates that early flowering enhances their reproductive success and may be under positive selection in these species. Because the reproductive success of food-deceptive orchids is mainly pollination-limited [11], pollinators probably select for this trend. Interestingly, many insects like honeybees, solitary bees, butterflies and moths, can learn associations between floral traits and reward, as do bumble-bee [21]. Therefore, GFD orchid species pollinated by these insects are likely to experience similar beneficial effects of early flowering owing to pollinator learning. Whether similar selection for long-lived flowers is widespread in GFD orchids remains unclear, because relevant investigations have yet to be conducted.

(b) Do associative and/or avoidance learning explain the higher pollinator visitation to early-flowering Calypso bulbosa?
Associative learning by bees for rewarding species probably contributed little to the higher pollinator visitation...
to early- compared with late-flowering *C. bulbosa*, and if anything may benefit the latter. Indeed, bumble-bees that had previously experienced *A. uva-ursi* approached and landed on more *C. bulbosa* flowers than naive bumble-bees, indicating that prior association of floral signals with reward increases the likelihood that a pollinator will investigate rewardless species. However, this effect did not persist to affect the incidence of flower probing and so did not alter pollination by bees that had experience with rewarding flowers compared with that by naive bees.

Our laboratory experiment instead strongly implicates avoidance learning as the explanation for the higher pollinator visitation to early- compared with late-flowering *C. bulbosa* observed in the field. Avoidance is evident in the reduced approaches, landings, proboscis extensions and probes of *C. bulbosa* flowers by bees during their second exposure to this species than during their first exposure. Additionally, because of the sequential dependence of behaviours during a visit, reduced frequency of early behaviours, such as approaches, had cascading effects for the incidence of latter behaviours, especially probes, which are necessary for pollination. Consequently, avoidance learning resulted in fewer pollinaria removals by experienced bees than by naive bees, which should affect male reproductive success. That pollen deposition was not affected similarly probably reflects the precise matching a butterfly-pollinated orchid.

The mechanism by which pollinators impose selection on flowering phenology of deceitful species may differ according to the floral traits of co-occurring rewarding species, including similarity of visual and olfactory signals. Dissimilar signals should hasten the development of discrimination caused by both associative and avoidance learning [1,20]. Similarity of signals should slow the development of negative associations through associative learning with rewarding species, but should not alter the impact of avoidance learning. Therefore, avoidance learning should act as a key mechanism promoting higher visitation and reproductive success in early-flowering deceptive species, regardless of their signal similarity to sympatric rewarding species. Associative learning may accentuate such selection in GFD orchids with signals that differ strongly from those of co-flowering rewarding species.

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