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Original article

Effect of nectar supplementation on male and female components of pollination success in the deceptive orchid *Dactylorhiza sambucina*

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ABSTRACT

Many orchids lack floral nectar rewards and therefore rely on deception to attract pollinators. To determine the effect that a mutation for nectar production would have on overall pollination success of the deceptive orchid *Dactylorhiza sambucina*, we recorded pollen deposition and removal in flowers of plants that had either been supplemented with an artificial nectar solution or left unmanipulated as controls. Nectar supplementation resulted in significant increases in the proportion of flowers pollinated, regardless of morph colour and the density of plants supplemented in the population. However, nectar supplementation had a significant positive effect on pollinaria removal only for the yellow morph in one experiment in which a low proportion of plants were supplemented. Thus a mutation for nectar production would have a positive effect on overall pollination success in *D. sambucina*, particularly the female component. The observed patterns are discussed in relation to other factors, such as cross-pollination and the reallocation of nectar resources for other plant functions, which are traditionally considered to shape the rewardless strategies of orchids.

1. Introduction

Pollen removal and deposition in most animal-pollinated plants depends on the presence of a reward such as nectar to entice pollinators (Simpson and Neff, 1983). When flowers are supplemented with nectar, pollinators tend to probe more flowers per inflorescence, which, in turn, tends to increase overall pollen export and import (cf. Mitchell and Waser, 1992; Mitchell, 1993; Burd, 1995; Johnson et al., 2004; but see Smithson, 2002). However, such behaviour may also result in higher levels of self-pollination, and thus cause pollen and ovule discounting when pollen available for export is lost to self-depositions, and ovules fertilized by self-pollen are lost due to inbreeding depression (Holsinger and Thomson, 1994; Herlihy and Eckert, 2002; Johnson et al., 2004).

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Some animal-pollinated plants lack floral rewards altogether (Heinrich, 1979; Bell, 1986). While rewardlessness has been recorded in several plant families, the phenomenon is most common among orchids (Renner, 2005). Approximately one-third of all orchid species (estimated as 6500 (Renner, 2005) to 8000 species (Dressler, 1981)), do not provide floral rewards, and rely on various forms of deception for pollination (Dafni, 1984; Ackerman, 1986; Nilsson, 1992; Jersáková et al., 2006a). Most orchids associated with food deception very often flower gregariously in early spring, exhibit floral colour polymorphism and exploit newly emerged bees and bumblebees after hibernation (Heinrich, 1975). Sometimes rewardless species benefit from growing in the vicinity of nectariferous co-flowering species, as these increase abundance of pollinators in the orchids' local habitat (vis a vis the magnet species effect: Laverty, 1992; Lammi and Kuitunen, 1995; Johnson et al., 2003). Pollinators are more likely to depart from areas with few flowers, either rewarding or rewardless, and conversely remain for longer in patches that provide a high reward or where rewarding flowers are highly aggregated (Real, 1983). The reproductive success of rewardless species thus strongly relies on the learning abilities of pollinators, which depend on relative co-flowering rewarding and rewardless plant species spatial distributions (Ferdy et al., 1998; Internicola et al., 2006).

Broad surveys suggest that pollination success is generally lower in rewardless orchids compared to rewarding ones (Dafni and Ivri, 1979; Gill, 1989; Johnson and Bond, 1997; Neiland and Wilcock, 1998; Tremblay et al., 2005). However, there have been few studies in which the consequences of nectar for pollination success have been determined by means of field experiments. Johnson and Nilsson (1999) found that nectar supplementation increased pollen deposition and removal in some, but not all populations of the orchids *Anacamptis morio* and *Orchis mascula*. Both pollen deposition and removal were significantly elevated by nectar supplementation in *Disa pulchra* (Jersáková and Johnson, 2006). Pollen deposition was not affected by nectar supplementation in *Barlia robertiana* and *Anacamptis morio* (Smithson and Gigord, 2001; Smithson, 2002). In a study of *Barlia robertiana*, nectar addition led to decreased pollinaria removal, leading Smithson and Gigord (2001) to propose that deception might actually increase the rate of removal of pollinaria from flowers. However, other studies have shown either no effect of nectar addition on pollinaria removal (Smithson, 2002) or a positive effect of nectar on pollinaria removal (Johnson and Nilsson, 1999; Johnson et al., 2004; Jersáková and Johnson, 2006). Because of the varying results of these studies, including differences in the outcome of nectar supplementation for male and female components of pollination success, there is a need for further studies that address the issue of how rewardlessness influences the pollination success of plants, including those with corolla-colour polymorphisms.

The aims of this study were to determine the effect of nectar supplementation for pollination success of the deceptive orchid, *Dactylorhiza sambucina* (L.) Soó, a non-nectariferous orchid known for its flower-colour polymorphism, with sympatric yellow- and purple-flowered individuals present in the same natural populations (Nilsson, 1980). Specifically, we asked (1) whether nectar supplementation would have differing consequences for male (pollen removal) and female (pollen deposition) components of pollination, and (2) whether the effects of nectar supplementation would differ between yellow and purple flower colour morphs. In addition, as reproductive success of rewardless plants is expected to be density-dependent, we tested (3) whether the density of plants supplemented in a meadow would have consequences for the pollination success of individual plants.

2. Materials and methods

2.1. *The study species*

Dactylorhiza sambucina (L.) Soó is a gregarious spring-flowering orchid, widely distributed throughout the Mediterranean, central Europe, and into southern Scandinavia. The plants have basal leaf rosettes and 10–30 cm tall stalks bearing a dense inflorescence of 5–20 flowers that are either yellow or purple. The two colour morphs are identical in plant height, number of leaves, flower number and size, and as far as known, also scent (Nilsson, 1980; Gigord et al., 2001, 2002; Kropf and Renner, 2005; Pellegrino et al., 2005; Jersáková et al., 2006b). The flowers' labellum (lip) provides a landing surface for bees and bears a basal 10–15 mm long empty spur. Pollen packages (pollinaria) are formed by the content of entire pollen sacs and are attached to pollinators via a viscidium and short stem. Pollen is deposited as clusters of pollen grains (massulae) that stick to the concave stigmas of the first couple of flowers visited by the pollinator.

Dactylorhiza sambucina is highly specialized for pollination by queen bumblebees (Nilsson, 1980). It blooms very early in the spring for 3–4 weeks in April–May, and therefore benefits from the naïveté of bumblebee queens emerging from hibernation. *D. sambucina* is pollinated in Europe mostly by *Bombus lapidarius* L., *B. terrestris* L., *B. pascuorum* (Scopoli) and *B. lucorum* L. (Nilsson, 1980; Gigord et al., 2001; Kropf and Renner, 2005; Jersáková et al., 2006b). Honeybees and solitary bees have been recorded as occasional visitors (Nilsson, 1980; Pettersson and Nilsson, 1983; Reinhard et al., 1991; Vöth, 1999; Pellegrino et al., 2005; Kropf and Renner, 2005). At our study site, *B. lapidarius* and *B. terrestris*, were the main pollinators (JJ, personal observation).

2.2. *Site description*

The experiments were performed during May 2003 in a large *D. sambucina* population consisting of several thousands individuals spread over the complex of dry pastures in the vicinity of the village Řetenice (49°07′N, 13°36′E), at the foothill of the Šumava mountains in the region of South Bohemia, Czech Republic. The overall relative morph frequency was 68% purple-flowered plants. There were no other nectariferous bumblebee-pollinated plants flowering intermingled with *D. sambucina*.

2.3. *Nectar supplementation*

Flowers were supplemented with a sucrose solution on a daily basis (even if the solution added on a previous day had not been consumed by pollinators). Every morning (between 9 and 11 a.m.), for 7 days, starting with the day when at least 5 flowers were open in both control and supplemented inflorescences, we injected 2 μl of the artificial nectar using a 10 μl Hamilton microsyringe into the spur of each flower of the supplemented inflorescence. We used 25% sucrose solution (by making 25 mg sucrose solution up to 100 ml total volume with distilled water), which is a compromise between being too diluted for the pollinators (bumblebees in our case) and a concentration leading to early flower wilting (Johnson and Nilsson, 1999). The experimental plants (both supplemented and control) were caged to exclude pollinators before we started the experiment.

2.4. *Experimental design*

In the first experiment, we selected plants over two meadows, A and B, to create a design with low density of nectar-supplemented plants (i.e., 1 supplemented plant per 100 m^2). The population sizes in the meadows A and B were 1064 and 1268, the relative frequencies of purple morphs were 58% and 77%, average plant densities (±SE) were 1.5 ± 0.34 and $1.6 \pm 0.34/m^2$ and the mean numbers of open flowers (±SE) were 11.6 ± 0.42 and 12.0 ± 0.61, respectively. In meadow A, we selected 25 pairs of plants with yellow inflorescences and in meadow B 22 pairs of plants with purple inflorescences. Plants that were paired were situated within 2 m of each other and were similar in terms of the number of open flowers (*t*-tests for dependent samples: yellow pairs: $t = -1.96$, d.f. = 23, $p = 0.06$; purple pairs: $t = 0.41$, d.f. = 20, $p = 0.68$). In each pair, one plant was supplemented daily, the other one was used as a control.

In the second experiment, we selected 2 patches (ca. 5 m radius) dominated by purple morphs in another meadow, C, where we created a design with a high density of nectar-supplemented plants (i.e., 15 supplemented plants per 100 m^2). The population size in the meadow C was 1050, the relative frequency of purple morphs was 69%, average plant density (±SE) was 1.2 ± 0.16 plants/m^2 and the mean numbers of open flowers (±SE) was 13.1 ± 0.59. In both of these patches, 15 purple morph plants were supplemented, and 9 and 15 purple morph plants respectively were used as controls. In both patches, the number of open flowers of supplemented plants was not different from that of control plants (*t*-tests for independent samples: patch 1: $t = 0.17$, d.f. = 21, $p = 0.86$; patch 2: $t = 0.88$, d.f. = 28, $p = 0.38$).

After one week of nectar supplementation, we recorded the number of open flowers per inflorescence, and, for each flower, the number of pollinaria removed and whether pollen had been deposited on the stigma.

2.5. *Data analysis*

The experimental plants, which experienced no pollen deposition or removal, were removed from the analyses. The total number of plants used in designs with low and high density of nectar-supplemented plants equalled 24 pairs of yellow morph and 21 pairs of purple morph, and 53 purple plants in patches, respectively.

Female pollination success was calculated as the proportion of flowers with pollinaria deposited (number of flowers with pollen deposition/number of open flowers), while male pollination success was calculated as the proportion of flowers with pollinaria removed (number of flowers with 1 or 2 pollinaria removed/number of open flowers). These data were tested for normality using the Kolmogorov–Smirnov test. The effects of nectar supplementation and reward density on pollination success were assessed using two-way ANOVA analyses with interaction terms, which were followed by paired *t*-tests to compare pollen removal and pollen deposition of supplemented and control plants within each combination of treatment (high and low density) and colour morph. In the first ANOVA, we tested the effects of nectar supplementation in a design that included morph colour as a factor. In the second ANOVA we tested the effect of supplementation in a design that included the density of supplemented plants as a factor. This second analysis was restricted to purple morphs as no yellow morphs were supplemented in the high supplemented plants density patches due to lack of such patches at our site.

3. Results

The results of two-way ANOVAs with interactions testing the overall effect of nectar addition on pollination success showed that nectar supplementation increased both pollen removal and deposition in both colour morphs of *D. sambucina* (see significant effects of supplementation and non-significant interactions, morph colour × supplementation in Table 1). However, when we performed detailed *t*-tests on each experimental group (see statistical significances in Fig. 1), nectar supplementation resulted in significant increases in pollen deposition in all treatment groups, pollen removal was significantly increased only in yellow morph in the experiment in which a low density of plants was supplemented with nectar (Fig. 1). Interestingly, we also found a significant effect of morph colour on both pollen removal and deposition: the yellow morph both received and exported nearly twice as much pollen as the purple morph (Table 1).

In the second experiment, where we tested the effect of density of nectar-supplemented plants (low versus high) and nectar supplementation on pollination success of the purple morph, supplementation significantly increased both male and female functions in purple morph plants of *D. sambucina* regardless of the density of supplemented plants (see significant effects of supplementation and non-significant interactions, density × supplementation in Table 2). Density of nectar-supplemented plants had a significant positive overall effect on pollen removal, but not on pollen deposition (Table 2).

4. Discussion

The results show that nectar supplementation significantly increased pollen deposition, regardless of morph colour and the proportion of plants supplemented in the population (Fig. 1, Tables 1, 2). Nectar supplementation also had

Table 1 – Results of two-way ANOVAs testing the effect of nectar supplementation and morph colour on the proportion of flowers with (a) pollinaria removed and (b) massulae deposited

	SS	DF	MS	*F*	*p*
(a) Proportion of flowers with pollinaria removed					
Intercept	14.041	1	14.041	358.431	0.000
Morph colour	1.176	1	1.176	30.024	**0.000**
Supplementation	0.300	1	0.300	7.673	**0.006**
Morph colour × supplementation	0.025	1	0.025	0.659	0.418
Error	5.445	139	0.039		
(b) Proportion of flowers with massulae deposited					
Intercept	24.219	1	24.219	541.506	0.000
Morph colour	1.868	1	1.868	41.781	**0.000**
Supplementation	1.157	1	1.157	25.885	**0.000**
Morph colour × supplementation	0.004	1	0.004	0.106	0.744
Error	6.216	139	0.044		

Table 2 – Results of two-way ANOVAs testing the effect of density of nectar-supplemented plants (low versus high) and nectar supplementation on the proportion of purple morph flowers with (a) pollinaria removed and (b) massulae deposited

	SS	DF	MS	*F*	*p*
(a) Proportion of flowers with pollinaria removed					
Intercept	4.880	1	4.880	158.453	0.001
Density	0.273	2	0.136	4.435	**0.014**
Supplementation	0.132	1	0.132	4.294	**0.041**
Density × supplementation	0.170	2	0.085	2.768	0.068
Error	2.741	89	0.030		
(b) Proportion of flowers with massulae deposited					
Intercept	8.632	1	8.632	214.942	0.001
Density	0.098	2	0.049	1.232	0.296
Supplementation	0.951	1	0.951	23.689	**0.001**
Density × supplementation	0.173	2	0.086	2.165	0.120
Error	3.574	89	0.040		

a generally positive effect on pollinaria removal, but this was significant in only one treatment group, in which a low proportion of plants were supplemented with nectar (Fig. 1). These results imply that a mutation for nectar production in *D. sambucina* would have a positive overall effect on pollination success, particularly its female component.

Our experiments did not control for the effect of inserting a needle into spurs of supplemented plants, but for the

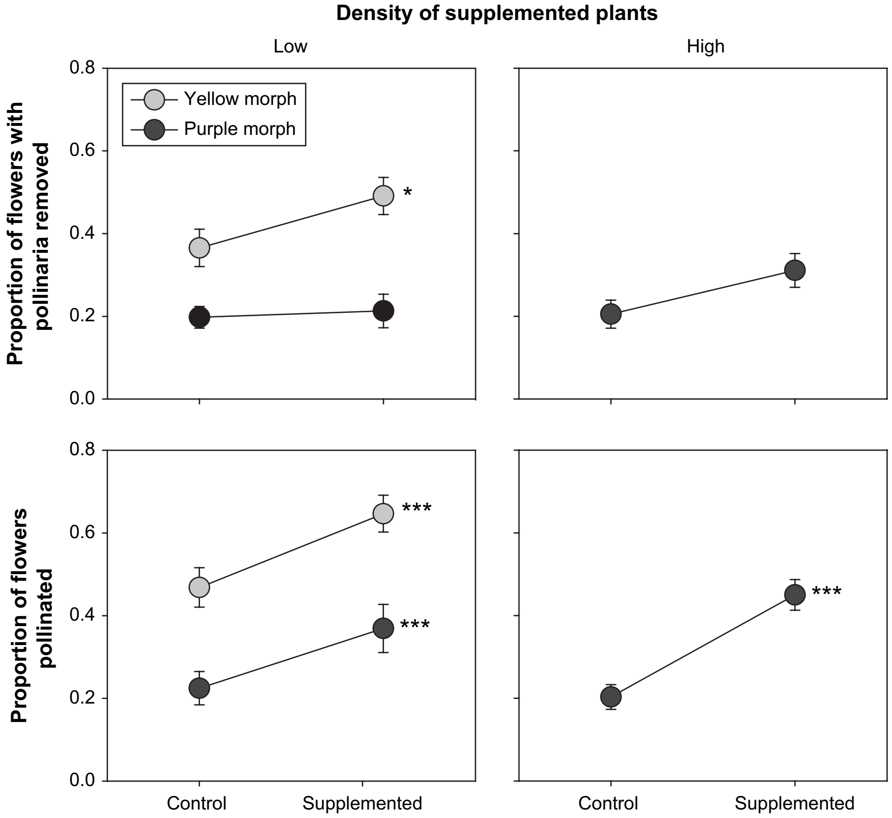

Fig. 1 – The effects of nectar supplementation on male and female measures of pollination success in two experiments varying in the density of nectar-supplemented plants. Symbols and error bars represent means and standard errors, respectively. The asterisks represent means that differed significantly between supplemented and control plants (paired *t*-tests performed on each colour morph at different densities; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). See Tables 1 and 2 for analyses of the overall effect of nectar supplementation in these experiments.

following reasons we do not think this would have had any influence on the results. First, the spur of *D. sambucina* is rather wide and we were able to insert the syringe without touching the reproductive organs, including the sensitive viscidium, which attaches the pollinium to the pollinator's body. Second, if nectar-supplemented flowers were damaged in some way by insertion of the needle, this could hardly explain their significantly higher levels of pollination success (Fig. 1).

The effects of nectar supplementation on pollination success of orchids have varied markedly among studies. Salguero-Faria and Ackerman (1999) found no effects of nectar supplementation on pollinaria removal and fruit set in *Comparettia falcata*, but this orchid, unlike *D. sambucina*, is a naturally rewarding species. Some studies found a marked increase in male and female components of pollination success (Johnson et al., 2004; Jersáková and Johnson, 2006), while others found that nectar supplementation had no effect on pollen deposition and a negative effect on pollen removal (Smithson and Gigord, 2001; Smithson, 2002). The discrepancies in the results might be caused by the way that experimental plants were offered to pollinators and how many times they were supplemented. Most of the above studies have used the "pollinator interview" approach whereby experimental inflorescences are presented to foraging insects. The present study is one of the few in which naturally growing plants were supplemented with nectar. Earlier attempts by Johnson and Nilsson (1999) to supplement naturally growing orchid plants with nectar yielded significant results in only some treatment groups. Unlike the present study, in which nectar was supplemented on a daily basis, Johnson and Nilsson (1999) supplemented plants just once during the flowering period, which may have weakened the power of their experiments.

The greater pollen deposition on stigmas of nectar-supplemented plants in our experiments may not necessarily translate into higher fitness. Other studies have shown that nectar supplementation increases pollen transfer among flowers on the same plant (geitonogamy), this being due to the higher number of flowers visited by pollinators (Johnson et al., 2004; Jersáková and Johnson, 2006). In general, self-pollination has negative consequences for plant fitness. First, it reduces pollen export and fewer ovules are successfully fertilized by outcross pollen, processes known as pollen and ovule discounting, respectively (Holsinger and Thomson, 1994; Herlihy and Eckert, 2002). Second, self-fertilization reduces levels of genetic variation (Charlesworth and Charlesworth, 1995) and may cause inbreeding depression, the existence of which has been demonstrated in *D. sambucina* (Juillet et al., 2006). Even though orchids are typically self-compatible, the seed set and seed quality is normally greatly reduced after self-pollination (Tremblay et al., 2005; Jersáková et al., 2006a).

Overall pollination success was always higher in yellow morphs, regardless of nectar supplementation. One explanation could be a conditioning effect whereby nectariferous co-flowering species, similar in flower colour and shape to the orchid may increase the probability that a pollinator will temporarily shift from a nectar-producing plant to a rewardless plant (cf. Johnson et al., 2003; Internicola et al., 2006). At our study site, there were no other co-flowering species visited by bumblebees flowering together with *D. sambucina* plants, contrary to other populations in the Czech Republic, where yellow (e.g. *Primula veris*) and/or purple (e.g. *Ajuga reptans*) nectariferous plants are frequently present (Jersáková et al., 2006b). However, bumblebees are capable of flying distances up to several hundred meters from the nest (Walther-Hellwig and Frankl, 2000) and thus might have been foraging on rewarding species in the vicinity of our study site. As a consequence, their preferences could be adjusted to plants at another site (spatial conditioning) and/or to previously visited plants (temporal conditioning) (Osborne et al., 1999; Burns and Thomson, 2006). An alternative possibility is innate pollinator preference for particular colours (Heinrich et al., 1977; Smithson and Macnair, 1997).

Experimental studies using bumblebees showed that a pollinator encountering a rewarding flower tends to decrease its flight distance to the next inflorescence, which would increase the probability of it visiting other individuals of the same phenotype (Dukas and Real, 1993; Smithson and Macnair, 1997; Cartar, 2004). The consequent expectation that overall pollination success would increase when there is a higher density of supplemented plants was supported only partially by our results, as we found a significant effect of higher density of supplemented plants on pollen removal but not deposition (Table 2). Furthermore, since the treatment of increasing the density of supplemented plants was limited to a single population, further studies involving multiple populations would need to be conducted to determine if these preliminary results can be generalized.

It is now increasingly clear, both from this study and several others mentioned above, that the absence of nectar from the flowers of many orchid species is detrimental to overall pollination success. Why then has a mutation for nectar production, as simulated by nectar supplementation in this study, not spread rapidly to fixation in populations of *D. sambucina* and other orchids? One possibility is that the mutations required for such a transition have not occurred (Gill, 1989). However, we know from phylogenetic studies that transitions from deceptive to rewarding pollination systems have occurred many times in orchids (cf. Johnson et al., 1998; Bateman et al., 2003). It seems more likely that there are real fitness advantages to deception, such as higher rates of cross-pollination and resource allocation, that outweigh the reduction in overall pollination success (Johnson et al., 2004; Jersáková and Johnson, 2006). Given that orchids are long-lived plants producing enormous amounts of dust-like seeds per capsule, a lower but continuous high quality fruit set can represent the best evolutionary stable strategy to ensure reproductive success during the orchid's lifetime (Cozzolino and Widmer, 2005).

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