

## REPRODUCTIVE SUCCESS AND SEX VARIATION IN NECTARLESS AND REWARDING ORCHIDS

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In hermaphroditic plants, the number of flowers in the inflorescence and the presence of a reward may have a differential effect on male (pollen dissemination) and female (pollen deposition) reproductive success and thus on the evolution of floral features. Two rewarding and one nectarless orchid species were used here to test the hypothesis that a large inflorescence serves primarily to increase male reproductive success (“male function hypothesis”). We have found that both in rewarding and in nectarless species, the rate of increase of male reproductive success with the number of flowers is not significantly different from that of female reproductive success, which does not support the male function hypothesis. Since the lack of reward causes lower reproductive success in nectarless orchids, we hypothesized that in nectarless species, the average time interval between flower opening and its first visit by a pollinator is longer compared with rewarding ones. Our data confirmed this hypothesis.

*Keywords:* reproductive success, reward, orchid, pollinator, gender, male function.

### Introduction

In terrestrial orchids, plant size, number of flowers, and presence of reward might affect both male (pollen dissemination) and female (capsule production) reproductive success (Bell 1985; Murren and Ellison 1996; Sabat and Ackerman 1996; Neiland and Wilcock 1998), but most studies equate reproductive success only with easy-to-measure capsule production (Nilsson 1980, 1984; Zimmerman and Aide 1989; Calvo 1990; Vallius 2000; Melendez-Ackerman and Ackerman 2001). However, pollen dissemination may be even more influenced by floral attractiveness compared with capsule production (Mitchell 1993; Smithson and Gigord 2001). According to the “male function hypothesis” (Willson 1979; Burd 1994), success in pollen dissemination increases with flower number, while the ratio of capsules produced/number of flowers in the inflorescence increases at a slower rate, levels off, or declines with increasing flower number because of resource limitation (Stephenson and Bertin 1983; Stanton et al. 1986; Campbell 1989). However, evidence for this in orchids is scarce and contradictory (Piper and Waite 1988; Waite et al. 1991; O’Connell and Johnston 1998). Here we analyze the effects of the number of flowers on male and female reproductive success in rewarding and nonrewarding terrestrial orchid species. We are testing the male function hypothesis by checking the assumption that male reproductive success increases at a larger rate with flower number than does female reproductive success. We also examine the previous suggestion (Nilsson 1992; Fritz 1995) that plants

with few flowers, which are supposed to be less attractive to pollinators, usually function as either males or neutrals, while plants with many flowers are hermaphrodites.

Nectarless orchids have a lower reproductive success than do rewarding ones (Neiland and Wilcock 1998), and the enormous floral longevity of nonpollinated flowers could have evolved because of low floral visitation rates (Calvo 1990). This led us to propose and test another new hypothesis. Since nectarless species do not offer any reward to their pollinators, it should, on average, take longer from flower opening to its first efficient visit by a pollinator (“time to first visit”) in nectarless species compared with rewarding ones. We use two rewarding species, *Gymnadenia conopsea* and *Platanthera bifolia*, with high fruit set and one nonrewarding species, *Dactylorhiza sambucina*, with low fruit set (89.3%, 93.1%, and 32.8%, respectively; Jersáková 1998) as model species.

### Methods

#### *Study Species and Sites*

*Gymnadenia conopsea* (L.) R.Br. and *Platanthera bifolia* (L.) L.C.Rich. offer nectar in long spurs, emit scent, and are pollinated predominantly by hawkmoths (Sphingidae: *Macroglossum stellatarum*, *Deilephila porcellus*) and moths (Noctuidae: *Cucullia umbratica*, *Autographa gamma*, *Autographa bractea*). A nectarless species, *Dactylorhiza sambucina* (L.) Soó, occurs in two color morphs (red and yellow) and is pollinated mainly by bumblebees (*Bombus lapidarius* and *Bombus terrestris*; van der Cingel 1995). Those particular orchid species were chosen because they occur in the same area.

The experiments were carried out at two adjacent sites in the northern part of the Sumava protected landscape area

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in Southern Bohemia, Czech Republic. The Retenice site (49°07'N, 13°36'E) is a south/southwest-facing downhill pasture at an altitude of 880 m a.s.l. with an abundant population of *D. sambucina*. The Javornik site (49°07'N, 13°39'E) has large populations of *G. conopsea* and *P. bifolia* growing in a south-facing hay meadow at an altitude of 890 m a.s.l.

### Field Experiments

At the beginning of the flowering season in 2000, we marked 50 plants of *G. conopsea*, 50 plants of *P. bifolia*, and 139 plants of *D. sambucina*. We followed the fate of all the flowers in these plants throughout their life span and recorded pollinia removal (male reproductive success), pollinia receipt, and capsule development (female reproductive success) every other day. We categorized flowers as pure male (one or two pollinia removed but no capsule produced), pure female (capsule produced but no pollinia removed), hermaphroditic (one or two pollinia removed and capsule produced), and neutral (no pollinia removed, no capsule produced). Since small quantities of pollen on stigmas could have been overlooked, we used the development of capsules rather than stigma pollination as the criterion for female reproductive success. Spontaneous autogamy and fruit abortion (pollinated flowers that did not set fruit) were recorded rarely (in *D. sambucina*, 2.0% of autogamy and 2.0% of fruit abortion; in *G. conopsea*, 3.2% of autogamy and 2.4% of fruit abortion; in *P. bifolia*, 1.5% of autogamy and 8.8% of fruit abortion). Consequently, fruit set closely tracked pollination and was a fair estimate of female reproductive success.

### Data Analysis

For each plant, we calculated the following values: (1) male reproductive success (the number of flowers with one or two pollinia removed) and female reproductive success (the number of developed capsules); (2) male (female) proportional reproductive success, calculated as male (female) reproductive success divided by the number of flowers produced; and (3) proportions of hermaphroditic, pure male, pure female, and neutral flowers out of the total number of flowers produced. We then divided the experimental plants into groups of plants with the same number of flowers and calculated averages of each of the calculated values for each

group. For each flower, we calculated (4) time to first visit as the number of days from the opening of a flower to its first visit by a pollinator, identified by either pollinia removal or receipt.

We used regressions to determine relationships between the averages of calculated values and number of flowers. We used quadratic regression; if the quadratic term was not significantly different from 0, we fitted the data by linear regression, and if neither the quadratic term nor the linear term was significantly different from 0, we fitted the data by a constant. The differences between linear regression curves were tested by ANCOVAs with reproductive success as the dependent factor, regression curve as the independent factor, and number of flowers as the continuous predictor. The data were checked for their normality and variance homogeneity to meet ANCOVA assumptions.

For each flower from which pollen was both exported and imported, we calculated the time span between the first pollinium removal and the first pollen receipt (considered as negative if pollinia removal preceded pollen receipt and vice versa). To analyze the interspecific differences in this value and in the time to first visit, we performed ANOVAs with subsequent post hoc comparisons (Tukey HSD for unequal sample sizes). Data were log transformed before the analysis. All analyses were performed using the statistical package Statistica ver. 5.5.

## Results

### Efficiency of Pollinators

In both rewarding species, the percentage of flowers visited by pollinators was approximately doubled compared with the rewardless species (table 1). However, the percentage of flowers in which removal of both pollinia occurred simultaneously with pollinia deposition was strikingly similar in all three species. We observed a higher percentage of pure male and pure female flowers in the rewardless species compared with the rewarding ones.

### Male and Female Reproductive Success

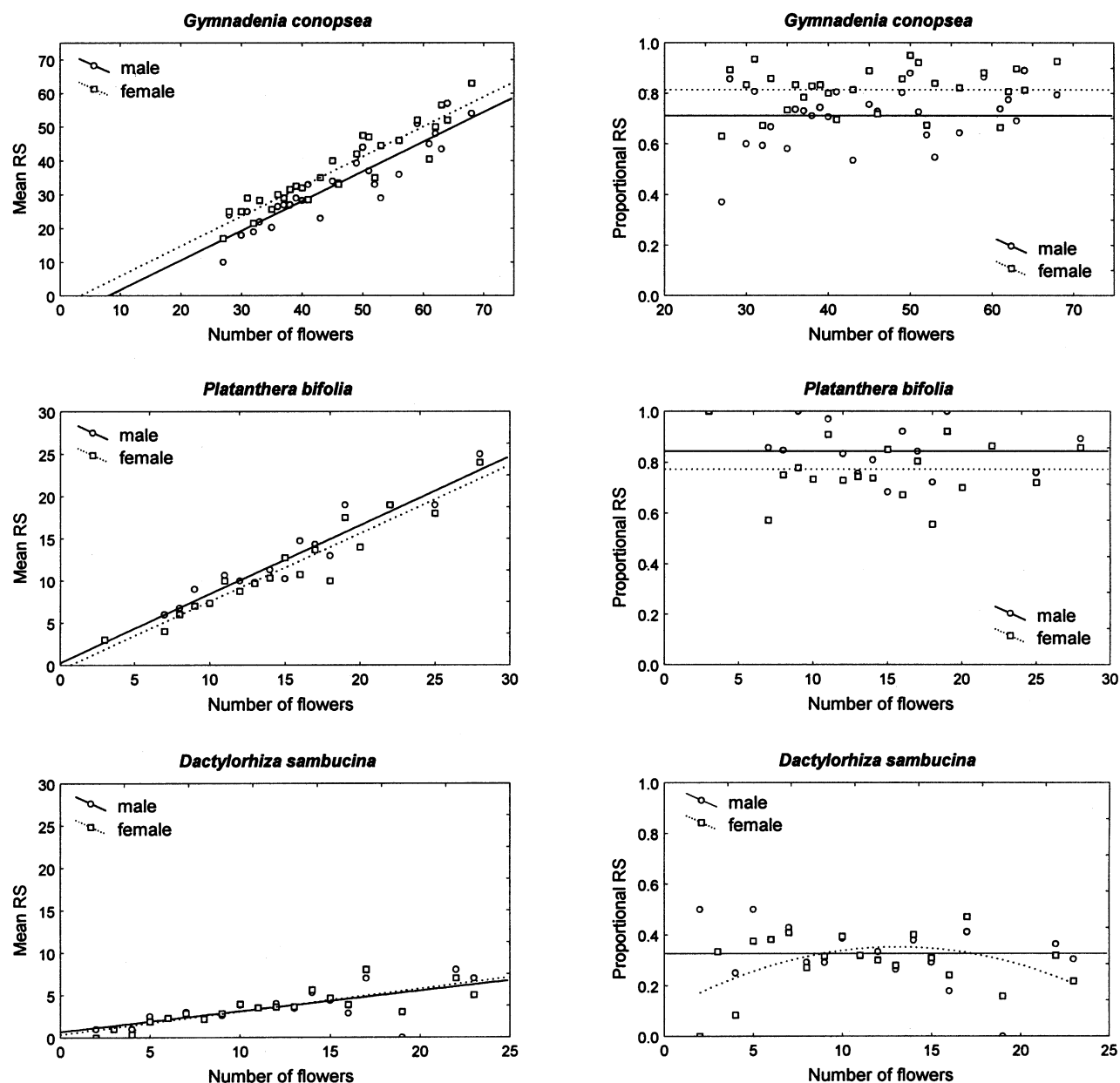
The number of successful male and female flowers increased linearly and almost always proportionately with the number of flowers in all three orchid species (fig. 1). Only

Table 1

Efficiency in Pollinia Removal and Receipt in Visited Flowers in Rewarding *Gymnadenia conopsea* and *Platanthera bifolia* and in Nectarless *Dactylorhiza sambucina*

Species	Percentage of flowers visited	Percentage of occurrences in visited flowers					
		1M and F	2M and F	M before F	F before M	Pure M	Pure F
<i>G. conopsea</i>	88.3	14.8	43.4	16.0	4.7	7.7	18.7
<i>P. bifolia</i>	92.3	20.0	46.2	13.0	1.6	17.1	10.4
<i>D. sambucina</i>	43.0	3.6	42.0	3.6	0.9	25.0	26.6

Note. 1M and F = simultaneous one-pollinium removal and receipt; 2M and F = simultaneous two-pollinia removal and receipt; M before F = pollinia removal occurred before receipt; F before M = pollinia receipt occurred before removal; pure M = flowers with only pollinia removal; pure F = flowers with only pollinia receipt.



**Fig. 1** Male and female reproductive success (RS; mean reproductive success on the left, proportional reproductive success on the right) in rewarding *Gymnadenia conopsea* and *Platanthera bifolia* and in nectarless *Dactylorhiza sambucina*. See table A1 for the regression equations.

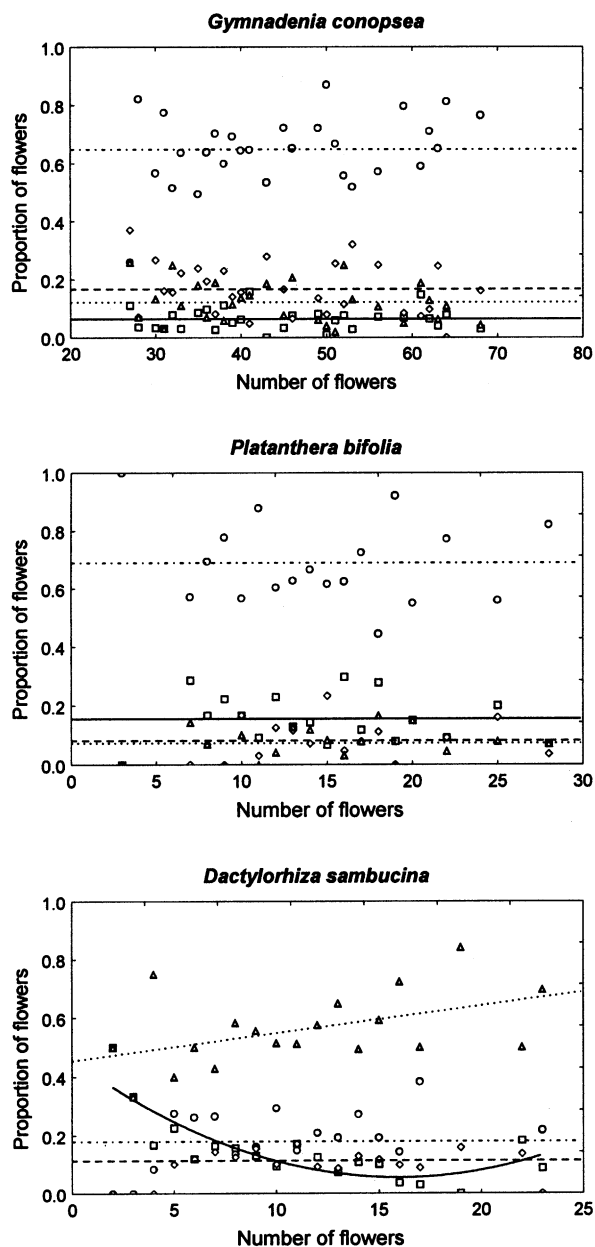
the female proportional reproductive success of *Dactylorhiza sambucina* has shown a weak negative (curved down) quadratic dependence on the number of flowers. While the slopes of linear curves for male and female reproductive success in both rewarding species were higher than 0.80, the slopes for *D. sambucina* were lower than 0.30 (see table A1 for the regression equations).

Independent of the number of flowers, pollen was predominantly received rather than removed in *Gymnadenia conopsea* ( $F_{1,53} = 13.96$ ,  $P < 0.001$ ), while in *Platanthera bifolia* and *D. sambucina*, the regression curves for pollinia receipt and removal did not differ significantly ( $F_{1,33} = 2.88$ ,  $P > 0.05$ ;  $F_{1,35} = 0.00001$ ,  $P > 0.05$ , respectively). The same

results were obtained for male and female proportional reproductive success in all three species ( $F_{1,53} = 14.55$ ,  $P < 0.001$ ;  $F_{1,33} = 3.80$ ,  $P > 0.05$ ;  $F_{1,35} = 0.80$ ,  $P > 0.05$ , respectively).

#### Pollinator-Generated Sexual Genders

The proportions of flowers that functioned as hermaphrodites, pure males, pure females, and neutrals are plotted against the number of flowers in figure 2. In *G. conopsea* and *P. bifolia*, most of the flowers were hermaphrodites: 65% and 67%, respectively (table 2). For both rewarding species, the proportions of flowers with a particular gender



**Fig. 2** Proportions of flowers with particular sexual functions in relation to the number of flowers in the inflorescence in rewarding *Gymnadenia conopsea* and *Platanthera bifolia* and in nectarless *Dactylorhiza sambucina*. Dotted and dashed line with circles = hermaphroditic (one or two pollinia removed and capsule produced), solid line with squares = pure male (one or two pollinia removed but no capsule produced), dashed line with diamonds = pure female (capsule produced but no pollinia removed), dotted line with triangles = neutral (no pollinia removed, no capsule produced). See table A2 for the regression equations.

were independent of the number of flowers. In *D. sambucina*, 57% of the flowers were neutral, unsuccessful in both pollinia removal and receipt (table 2), and their proportion was positively associated with the number of flowers. The proportions of hermaphroditic and pure female flowers did not depend on the number of flowers; the number of pure

male flowers had a positive quadratic (curved up) character with higher proportions in plants with fewer than five flowers.

#### *Time to First Visit*

The first efficient visit occurred significantly later in *D. sambucina* than in *G. conopsea* and *P. bifolia* (table 3). The mean differences between the day of first pollinia removal and the day of first pollinia receipt were  $-0.48$  and  $-0.50$  d for rewarding *G. conopsea* and *P. bifolia*, respectively, while only  $-0.13$  d (approximately simultaneous) for deceptive *D. sambucina* (table 3). The first pollinia were usually received 2–5 d after flower opening and removed 3–8 d after flower opening in all three species. During the first 2 d after flower opening, pollinia were removed from 19% of flowers, and stigmas of 11% and 15% of the flowers were pollinated in both rewarding *G. conopsea* and *P. bifolia*. In *D. sambucina*, these values were 11% and 10%, respectively. In some cases, pollinia were removed or stigmas pollinated as late as 2 wk after flower opening in each of the three species.

#### **Discussion**

##### *Influence of Inflorescence Size*

In hermaphroditic plants, size of floral display (measured as the number of flowers in the inflorescence) may have a differential effect on the male (siring seeds) and on the female (setting seeds) side of reproduction. Both male and female fitness can be a decelerating, linear, or accelerating function of the number of flowers. If there were a fitness advantage in large floral display plants, then one would expect male and female proportional reproductive success to increase with the number of flowers. Since the orchid reproduction can be resource limited over its lifetime (Melendez-Ackerman et al. 2000), the female proportional reproductive success may be negatively associated with the number of flowers for large inflorescences. Thus, the large inflorescence size would be selected mainly through pollen dissemination (male function hypothesis; Campbell 1989).

According to our observations, in nectariferous species *Gymnadenia conopsea* and *Platanthera bifolia*, male and female proportional reproductive success were constant and not significantly different from each other, which does not support the hypothesis that large inflorescence size serves primarily to increase male function (Waite et al. 1991). The number of flowers of nectariferous plants seems to be limited by resource availability in the preceding season (long-term resource limitation; Melendez-Ackerman et al. 2000) rather than by pollinator limitation.

In deceptive *Dactylorhiza sambucina*, the proportion of flowers that developed fruits had a weak negative (curved down) parabolic shape; i.e., female reproductive success was smallest in small and large floral display plants. This type of dependence, which has also been found in *Orchis morio* (Jersáková and Kindlmann 1998), would indicate that plants with a small number of flowers are too inconspicuous to be found by pollinators repeatedly, while in plants with a large number of flowers, low visitation rate combined with low

**Table 2**  
**Pollinator-Generated Sexual Function in Rewarding *Gymnadenia conopsea* and *Platanthera bifolia* and in Nectarless *Dactylorhiza sambucina***

Species	Total number of flowers	Percentage that functioned as			
		Pure male	Pure female	Hermaphroditic	Neutral
<i>G. conopsea</i>	2203	6.8	16.5	65.0	11.7
<i>P. bifolia</i>	666	15.8	9.6	67.0	7.7
<i>D. sambucina</i>	1434	11.1	11.4	20.4	57.0

Note. Percentage of flowers with pure male (one or two pollinia removed but no capsule produced), pure female (capsule produced but no pollinia removed), hermaphroditic (one or two pollinia removed and capsule produced), and neutral (no pollinia removed or capsule produced) functions.

number of flowers pollinated per visit negatively affects fruit set. In other deceptive species, *Calopogon tuberosus* (Firmage and Cole 1988), *Aspasia principissa* (Zimmerman and Aide 1989), and *Dactylorhiza maculata* (Vallius 2000), the proportion of flowers that develop into fruit was found mostly to be independent of floral display.

Large number of flowers in both rewarding and deceptive species seems to be advantageous in favorable years, “ecological windows,” because resources and pollinators occur unpredictably in time or space (the bet-hedging hypothesis; Sutherland 1986). According to our observations, female proportional reproductive success was strongly dependent on year and site in several orchid species (J. Jersáková and P. Kindlmann, unpublished data); only in particular years did large plants develop proportionately (linear trend) or disproportionately (nonlinear trend) more fruits than small plants, while in other years no trend was observed. Two studies on another rewarding species, *Epipactis helleborine*, show a similar phenomenon: linear (Piper and Waite 1988) or no significant relationship between the proportional fruit set and inflorescence size (Waite et al. 1991).

Pollinia removal has been reported to occur predominantly before receipt (Piper and Waite 1988; Waite et al. 1991; Fritz 1995). However, in the species we investigated, pollinia loss occurred simultaneously with pollinia receipt in about 50% of pollinator visits (table 1). Much less frequently were either pollinia removed before pollinia receipt or pollinia received before their removal. These values were especially low in *D. sambucina*, probably because of fewer visits per flower in deceptive species (O’Connell and Johnston 1998).

Reproductive success of an individual is often dependent on its size, which is particularly true in plants, and natural

selection ought to favor a change in sex function—from small male plant to large female or cosexual one—as a plant grows and can afford to allocate resources to costly seed production (Ghiselin 1969). In our observations, proportions of flowers that function as hermaphrodites, pure males, pure females, and neutrals were independent of the number of flowers, and the flowers were mostly hermaphroditic in both rewarding species. Quite a different situation appeared in deceptive *D. sambucina*, where most flowers were neutrals, with their number being positively associated with the number of flowers. In individuals with fewer than five flowers of *D. sambucina*, a large proportion of flowers functioned as pure males, which is consistent with Nilsson’s (1992) results on *Orchis morio* and Fritz and Nilsson’s (1994) results on three nectarless species, *Anacamptis pyramidalis*, *Orchis palustris*, and *Orchis spitzelii*. However, the proportion of hermaphroditic flowers was independent of the number of flowers and thus in contradiction with the aforementioned studies, where many-flowered individuals were mostly hermaphrodites. Flowers of both rewarding species, *G. conopsea* and *P. bifolia*, were predominantly hermaphrodites, which is not surprising since their reproductive success is very large, but their proportions did not increase with increasing number of flowers. Therefore, we disagree with Fritz (1995) that many-flowered individuals have a more stabilized hermaphroditic gender than few-flowered plants, a pattern that is supposed to be universal in populations of pollinator-limited angiosperms. The reason why our results differ from those of Fritz and Nilsson (1994) may be that the authors have used functional sex on the basis of pure gender categories; i.e., a plant was classified as male when it had  $\geq 1$  pollinium removed but no fruit set and so on,

**Table 3**

**Mean  $\pm$  SD of Time to First Visit and Difference in Timing of Sex Functions in Flowers in Rewarding *Gymnadenia conopsea* and *Platanthera bifolia* and in Nectarless *Dactylorhiza sambucina***

Variable	Mean $\pm$ SD		
	<i>G. conopsea</i>	<i>P. bifolia</i>	<i>D. sambucina</i>
Time to first visit (d)	3.7 $\pm$ 0.8 <sup>A</sup>	3.7 $\pm$ 1.3 <sup>A</sup>	4.4 $\pm$ 2.4 <sup>B</sup>
Difference between the day of first pollinium removal and the day of first pollen receipt (d)	-0.48 $\pm$ 0.28 <sup>A</sup>	-0.50 $\pm$ 0.66 <sup>A</sup>	-0.13 $\pm$ 0.62 <sup>B</sup>

Note. Different letters mean significant differences at  $P < 0.05$ , Tukey HSD test.

whereas we used functional sex on the basis of the proportion of flowers with a particular sex per individual. If we used their approach, we would have recorded no pure male, pure female, or neutral plants in rewarding species at all. In *D. sambucina*, we found 11 pure male, six pure female, three neutral, and 119 hermaphroditic plants. Thus, if we used Fritz and Nilsson's (1994) approach, we would have obtained the same results: pure male and neutral plants have significantly fewer flowers (7.4 and 5, respectively) than do pure female and hermaphroditic plants (10.8 and 11.2, respectively;  $F_{3,125} = 6.16$ ,  $P < 0.001$ ). We consider our approach more biologically meaningful, since individual plants are rarely pure in their sex but, rather, a mixture of flowers with various functional genders, which vary in their proportions.

#### Time to First Visit

We confirmed that a longer period of time elapses before the flowers of the nectarless species receive or lose pollinia than in rewarding species. In the rewarding species, the average instant of pollinia receipt was always later than that of pollinia removal. This was more obvious in *G. conopsea*, where immediately after flower opening the stigma is not sticky and pollinia do not adhere to its surface. Therefore, pollinia removal occurred about half a day earlier than pollinia receipt. This is probably caused by the higher revisita-

tion rate in rewarding species, which is well illustrated by the percentage of pollinia removed that occurred before pollinia receipt (13%–16% in rewarding vs. 4% in deceptive species, respectively; table 1). In the nectarless species, the timing of pollinia removal almost coincided with pollinia receipt. This indicates that in flowers that experienced both sexual functions, the pollinia receipt and removal occurred mostly during the same visit (table 1). The high numbers of pure male and pure female flowers refer to low revisitation rate (cf. tables 1, 3).

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### Appendix

Table A1

Regression Parameters of Fitted Curves for Male (m) and Female (f) Reproductive Success (RS; Mean and Proportional) in Relation to Number of Flowers in Rewarding *Gymnadenia conopsea* (GC) and *Platanthera bifolia* (PB) and in Nectarless *Dactylorhiza sambucina* (DS)

	Regression	<i>a</i>	<i>b</i>	<i>c</i>	$R^2$	<i>F</i> -test	<i>P</i>
Mean:							
GC:							
mRS	Linear	0.877	-7.022		0.83	136.44	0.001
fRS	Linear	0.884	-2.959		0.87	186.62	0.001
PB:							
mRS	Linear	0.816	0.250		0.92	184.84	0.001
fRS	Linear	0.809	-0.568		0.91	165.85	0.001
DS:							
mRS	Linear	0.242	0.671		0.45	15.86	0.001
fRS	Linear	0.270	0.352		0.65	35.15	0.001
Proportional:							
GC:							
mRS	Constant			0.711			
fRS	Constant			0.814			
PB:							
mRS	Constant			0.844			
fRS	Constant			0.772			
DS:							
mRS	Constant			0.326			
fRS	Quadratic	-0.001	0.038	0.105	0.16	5.11	0.038

Note. *a*, *b*, *c* = parameters;  $R^2$  = coefficient of determination; *F*-test is the calculated *F* value, and *P* is the corresponding probability value.

Table A2

Regression Parameters of Fitted Curves for Proportions of Flowers with Particular Sexual Function in Relation to Number of Flowers in Rewarding *Gymnadenia conopsea* (GC) and *Platanthera bifolia* (PB) and in Nectarless *Dactylorhiza sambucina* (DS)

	Regression	<i>a</i>	<i>b</i>	<i>c</i>	<i>R</i> <sup>2</sup>	<i>F</i> -test	<i>P</i>
GC:							
Hermaphroditic	Constant			0.648			
Pure male	Constant			0.064			
Pure female	Constant			0.167			
Neutral	Constant			0.122			
PB:							
Hermaphroditic	Constant			0.690			
Pure male	Constant			0.155			
Pure female	Constant			0.082			
Neutral	Constant			0.073			
DS:							
Hermaphroditic	Constant			0.179			
Pure male	Quadratic	0.002	-0.051	0.459	0.68	15.98	0.001
Pure female	Constant			0.113			
Neutral	Linear	0.009	0.454		0.17	4.64	0.045

Note. *a*, *b*, *c* = parameters; *R*<sup>2</sup> = coefficient of determination; *F*-test is the calculated *F* value, and *P* is the corresponding probability value.

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