

On the evolution of protandry and the distinction between preference and rank order in pollinator visitation

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ABSTRACT

Question: How can protandry of hermaphrodite flowers be an adaptive strategy? Does this differ for plant species with vertical (*Digitalis purpurea*) and non-vertical (*Echium vulgare*) inflorescences?

Mathematical methods: We develop a measure for quantifying rank order of visitation to flowers in the male and female stage.

Key assumptions: Protandry is adaptive when it leads to female flowers being visited before male flowers.

Conclusions: In *D. purpurea*, female flowers were visited first; for a bumblebee visiting 10 flowers on the plant, the average rank of the female flowers visited was 2 rank numbers below that of the male flowers, while the maximum rank difference (all female flowers visited before male flowers) was 5. In *E. vulgare*, there was no consistent difference in rank of visitation, despite a strong preference of bumblebees for visiting high-rewarding male-phase flowers. While results for *Digitalis* are in line with expectation, those for *Echium* are not.

Keywords: bumblebee, dichogamy, Darwin's pollination syndrome, *Digitalis purpurea*, *Echium vulgare*.

INTRODUCTION

In many species with hermaphrodite flowers, male and female parts do not mature at the same time. The botanical term for this phenomenon is intrafloral dichogamy. Flowers are female first and then male (protogynous), or male first and then female (protandrous). Darwin (1862) argued that temporal separation of male and female parts reduces the selfing rate. This concept was widened after the discovery that many dichogamous species,

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especially those with protandry (Routley *et al.*, 2004), are in fact self-incompatible (Lloyd and Webb, 1986; Bertin and Newman, 1993). This suggests that apart from lowering the selfing rate, dichogamy must have other associated advantages. Two hypotheses have been put forward. First, dichogamy can increase seed set by reducing interference between pollen and pistils, even in species with 100% outcrossing. Second, dichogamy may increase pollen export to other plants when pollinators visit female flowers first and leave the plant after visiting male flowers (Darwin, 1862, p. 114; Bertin and Newman, 1993; Rademaker *et al.*, 1997; Routley and Husband, 2005).

One would expect that protogyny is the best solution to the selfing problem. A flower that starts off as female can be cross-fertilized by pollen from other individuals in the population. Should fertilization fail, then, as a second-best option, self-pollination occurs after the anthers ripen. Natural selection can adjust the time between the start of the female and male phase and, in this way, the period that the plant 'waits' for outcross pollination. If the plant was protandrous and pollen removal rates were low, the remaining pollen could well spoil the production of outcross seeds. Moreover, an animal-pollinated plant has no way of detecting how much of the pollen is already removed, so when the flower is protandrous entering the female phase is always a venture. This argument suggests that protogyny is the superior strategy to prevent selfing and it is a problem to explain why any species would be protandrous.

While wind-pollinated species with hermaphrodite flowers are indeed mostly protogynous (Bertin and Newman, 1993), the opposite pattern occurs among animal-pollinated hermaphrodites. Intrafloral protandry is more common than protogyny (865 vs. 437 species) and occurs in particular among species with vertical inflorescences visited by bees (Bertin and Newman, 1993). How can protandry be adaptive in such plant species?

In his observations on the orchid *Spiranthes autumnalis* (now *S. spiralis*) and the bumblebees that pollinate it, Darwin (1862, p. 114) emphasized that *Spiranthes* is well adapted through a combination of: (1) an acropetalous, vertical inflorescence in which flowers at the bottom open before flowers at the top; (2) protandrous flowers; and (3) pollinating insects that move from bottom to top. McKone *et al.* (1995) referred to this combination of characters as 'Darwin's syndrome'. Darwin proposed that this combination of characters results in insects cross-fertilizing the female flowers that they visit first, while flying off with newly collected pollen from the male flowers.

The latter would also occur if the plant flowers from top to bottom (basipetalous) and flowers were protogynous. However, no species with this set of characters has been reported. Lloyd and Webb (1986) mentioned *Cynoglossum officinale* as a possible example but this is incorrect, since all boraginaceous plants flower along cymes (i.e. along non-vertical inflorescences) and when the plant is in full bloom there is no obvious bottom-to-top gradient in flower age. McKone *et al.* (1995) documented that *Besseya bullii* combined acropetalous flowering with protogyny.

Some recent experimental studies demonstrated the adaptive value of Darwin's syndrome. By comparing protandrous with adichogamous inflorescences in *Chamerion angustifolium*, Routley and Husband (2003) showed that protandrous plants were twice as successful in exporting pollen. In a similar comparison in an orchid, Jersakova and Johnson (2007) showed that protandry greatly reduced deposition of self-pollen within the plant.

It has been suggested that pollinators visit more rewarding (female) flowers at the bottom first and leave the plant after visiting less rewarding (male) flowers at the top. There is some confusion on this issue in the literature. Pyke (1978) studied bees visiting *Delphinium nelsoni*, *D. barbeyi*, and *Aconitum columbianum*, three species that all fit Darwin's syndrome. After

bagging flowers for 24 h, Pyke found that nectar standing crop in the older, female flowers was highest. From this Pyke (1978) concluded that bees forage optimally by starting at the high-rewarding flowers at the bottom and then moving upwards, leaving the plant when the expected gain within the patch (plant) is lower than elsewhere. Best and Bierzychudek (1982) worked along similar lines, bagging flowers on *Digitalis purpurea* plants for 24 h. They also observed a gradient from high nectar reward at the bottom to low reward at the top. Charnov (1982, p. 259) concluded from Pyke's data that this suite of characters provides the plant species with an effective outcrossing mechanism, while maximizing pollen export. This is similar to Darwin's suggestion, but with the addition that bees forage optimally by visiting high-rewarding flowers first.

There are major problems, however, with the addition of Pyke (1978). Indeed, many pollinators like bees and hawkmoths typically forage from bottom to top on the inflorescence, but it is less clear that they visit the high-rewarding flowers first and then leave when rewards become too low. Without going into detail, Lloyd and Webb (1986) commented: 'The upwards movements of pollinators are often described as optimal foraging strategies, but the direction of movement does not always correspond with the postulated gradient of rewards' (p. 153). Lloyd and Webb (1986) further outlined that there are obvious reasons for pollinators to forage from bottom to top; these reasons include minimizing foraging costs, better flight control, better view, and reducing predation. One might also question whether the nectar production rate is really high at the bottom and low at the top as Pyke (1978) and Best and Bierzychudek (1982) claimed. These authors used plants that were bagged for 24 h. Male flowers were probably still closed at the time of bagging and not producing any nectar, while female flowers were open and producing nectar during the whole bagging period. Consequently, the high nectar standing crop in female flowers 24 h after bagging does not prove that female flowers have a higher nectar production rate.

Contrary to the claim of high nectar production in female flowers, many researchers have reported the highest nectar production rate when flowers are in their male stage (see Carlson, 2007, and references therein). High nectar in the male phase is in line with Bell's (1985) principle that in general more visits per flower are required for pollen removal than for fertilization. When male flowers are associated with high nectar, then, according to Pyke (1978), optimal foragers would visit the male flowers first, before moving on to the female flowers and leaving the plant when reward becomes too low. This behaviour would result in high self-pollination and low pollen export, which is clearly non-adaptive. So the question is: How is nectar production related to Darwin's syndrome and how does it affect pollinator movement?

All species mentioned until now have vertical inflorescences, with old flowers at the bottom and new flowers at the top (for further examples, see Lloyd and Webb, 1986), and they all seem to fit Darwin's syndrome. However, many protandrous species have non-vertical inflorescences and this group has hardly been studied in the present context (Lloyd and Webb, 1986). Such species flower along sub-branches (i.e. non-vertical inflorescences), with young male flowers more peripheral than old female flowers. For instance, all species in the Boraginaceae flower along non-vertical cymes with no clear bottom-to-top gradient along the main stem of the flowering plant. In such 'unstructured' inflorescences, pollinators cannot easily follow a bottom-to-top gradient and need to switch many times between male and female and low- and high-rewarding flowers. What is the function of protandry in these non-vertical inflorescences? How is bee visitation in this case affected by reward?

To address these questions, we need a measure for the rank order in which female and male flowers are visited. We distinguish between (1) the preference that pollinators have for

male-stage vs. female-stage flowers and (2) the rank order in which they visit these flowers. These two related concepts are fundamentally different. For example, imagine a pollinator visiting a plant with equal numbers of female (0) and male (1) flowers. Foraging bouts of 1000 or 0001 (listed from first to last) both show the same preference for female flowers but differ in the rank order of visitation. McKone *et al.* (1995) quantified the rank order of visitation by recording male–female and female–male transitions. Female–male transitions are better than the reverse. The disadvantage of the method is that it loses information on the total foraging bout. A 00010 bout would count the same as 01000, while the former would be more beneficial to the plant. We therefore develop a new method for ranking visits to male and female flowers. We illustrate the method with observations of bumblebee foraging on common foxglove (*Digitalis purpurea*) and viper’s bugloss (*Echium vulgare*), both species with markedly protandrous flowers. As noted above, the vertical inflorescence of *D. purpurea* has a clear structure with old female flowers at the bottom and young male flowers at the top. In *E. vulgare*, flowers open along cymes (i.e. from the inside of the plant to the outside), with no clear vertical gradient in the age of flowers or the associated nectar production.

METHODS

Potted flowering plants of *Digitalis purpurea* and *Echium vulgare* were placed in the field between 10 and 17 June 2010. Most bumblebees observed foraging on the two species were *Bombus terrestris* and *B. pascuorum*.

The *Digitalis* plants were uniform in size and had between three and five inflorescences. Bees, therefore, were able to drop after reaching the top on one inflorescence and continue with the bottom (female) flower of the next inflorescence on the same plant. Nectar was measured in *D. purpurea* flowers with 10 μ l microcapillaries. We measured nectar content in open flowers with continuous visitation. We also placed plants in a netted cage at 17.00 h and measured nectar at 09.00 h on the following day. Observations on bout length were made on 4 days for each species and we used only bout lengths of six flowers or more for analysis.

For *Echium vulgare*, we did not measure nectar. However, Corbet (1978) and Klinkhamer and de Jong (1990) previously showed that nectar production rate is higher in young male flowers, and Rademaker *et al.* (1999) observed a strong preference of bumblebees for these male flowers. Flower colour changes from pink to blue in *E. vulgare*. Bees may therefore use colour as a cue. Several observations on *E. vulgare* suggested that bees also assess nectar before entering the flower. Bees briefly halt before entering a flower or passing on to the next one (Rademaker and Taal, 1998). Bumblebees rarely enter a young flower of *E. vulgare* filled with (scentless) sugar water. Bees frequently visit old female flowers that have been bagged for some time (nectar accumulation), while the same flowers are avoided when the nectar has been depleted (T.J. de Jong, unpublished data).

For quantifying rank order of female and male flowers visited, we calculated the score D as follows. We record the rank order in which a bee visits two flower types (e.g. male/female flowers). For a bee that makes a foraging bout to six flowers on the same plant, this bout is denoted as 001001, where, as before, 0 denotes a visit to a female-stage flower and 1 a visit to a male-stage flower. Next, we calculate the average rank of the visited male (A_m) and female flower (A_f), which is the sum of ranks divided by visits to male and female flowers, respectively. In the example, $A_f = (1 + 2 + 4 + 5)/4 = 3$ and $A_m = (3 + 6)/2 = 4.5$. A positive

difference $A_m - A_f$ shows that the bee visits female flowers first, whereas a negative difference shows that the bee visits male flowers first. The value of the difference depends on bout length n and since this varies widely between different bee approaches, it is useful to control for this. Scaling the difference relative to the bout length (i.e. dividing the difference by n) does the trick. To give the measure D some intuitive meaning, we scale with respect to bout length of 10 flowers. D is then the difference in average rank between the male and female flowers visited on a plant with 10 flowers:

$$D = 10(A_m - A_f)/n.$$

Both flower types need to be visited before D can be calculated; thus all male or all female sequences are removed from the analysis. D varies between -5 and $+5$. Note that D measures only in what rank order flower types are visited and provides no information about which flower type is visited more often. For instance, extremes with bout length $n = 10$, such as 0000000001, 0000000011 or 0000000111, all result in an average rank difference of 5 and therefore $D = 5$. The scaling makes D independent of bout length, thus shorter bouts (e.g. 01 or 0011) or longer bouts in which all female flowers are visited before the male flowers also result in $D = 5$. Observe that for any bout with value D , the reversed (order) bout will have value $-D$; for example, for 001001, $D = 4.5 - 3.0 = 1.5$, while for the reversed bout 100100, $D = 2.5 - 4.0 = -1.5$. This implies that, for symmetrical sequences, where the original and reversed bouts are identical, D is always zero. When examining a large set of random visitation sequences in which male flowers appear with probability p , the average D will be zero, because each bout and its reverse will appear with the same probability. Moreover, because for any bout the reversed bout will appear with the exact same probability, the distribution of D values will be symmetric around zero. Monte Carlo simulations further showed that the symmetrical distribution of D around zero fits well to a normal distribution when $n > 10$.

RESULTS

On *D. purpurea* flowers, median nectar standing crop was typically higher on male flowers (0.20 μ l) than on female flowers (0.08 μ l) (Mann-Whitney test: $P = 0.03$, $n_1 = n_2 = 18$). After placing plants overnight in a tent, the pattern reversed and the nectar accumulated in female flowers (0.96 μ l) was at the border of significance higher than in male flowers (0.65 μ l) (Mann-Whitney test: $P = 0.07$, $n_1 = 15$, $n_2 = 17$). Averaged over the whole data set, female flowers received $\sim 15\%$ fewer visits per flower than male flowers.

We analysed 52 bee approaches over the complete observation period of 4 days. D was on average 1.97 with a 95% confidence limit of 0.79 and therefore significantly higher than one. Thus, bees tend to visit female flowers before visiting male flowers in *D. purpurea*. As noted previously (Best and Bierzychudek, 1984), the foraging pattern of the bees on *Digitalis* is typically from bottom to top.

In line with previously published results on *E. vulgare*, bees markedly preferred to visit male flowers over female flowers on all days. Overall, a male flower received 3.6 times more visits than a female flower and the pattern was consistent over the 3 days of observation (3.91, 3.58, and 3.27, respectively). There was no clear trend in D when comparing different days. On the first day, the average value of D was greater than zero, suggesting that bees visit female flowers first (Table 1). On the second and third observation

Table 1. *D*-values for 11 *Echium vulgare* plants measured on various days

Plant No.	Day 1	Day 2	Day 3
1	0.89 ^a	-0.16	-1.17
2	2.70*	0.42	—
3	-0.87	0.58	0.86
4	2.25	-0.72	0.08
5	2.10	1.28	-0.16
6	0.64	0.28	1.49*
7	1.44*	-1.39	0.59
8	1.41	1.05	-0.24
9	—	0.90	-0.60
10	1.38	0.12	0.88
11	—	0.13	-0.54
Mean (S.E.)	1.33 (0.35)	0.23(0.23)	0.12 (0.26)

^a Values are means of various (between 2 and 13) bout lengths on the same plant.

* $P < 0.05$ in a single-sample *t*-test comparing mean value of *D* against zero.

day, this trend was not observed. *D* was not significantly different from zero (i.e. bees visited male and female flowers in random order, despite their strong preference for entering male flowers).

DISCUSSION

For *D. purpurea*, we reproduced the well-known result of Best and Bierzychudek (1982) that bees move from bottom to top of the plant and therefore visit female flowers first followed by male flowers before leaving. In our experimental set-up, however, bees went against the nectar gradient, i.e. they began with the lowest rewarding female flowers at the bottom and finished with the most rewarding male flowers at the top. In *Digitalis*, protandry has the benefits outlined in the Introduction. Note, however, that the average difference in rank between male flowers and female flowers is only -2 , which is far from the ideal pattern for the plant when bees always visit female flowers first ($D = 5$). If we had used *Digitalis* plants with a single inflorescence, bees would have had no opportunity to switch between inflorescences and the pattern would probably have been closer to the ideal.

Despite a strong preference for male flowers, there was a slight (1.3 rank difference in a bout length of 10) tendency for female flowers to be visited before male flowers on the first day of observation in *E. vulgare*. This may reflect the tendency of bees to visit cymes from inside out. On both other days, no difference in rank of visitation was observed. This result is unsurprising if we accept that bees forage from bottom to top. On the other hand, the result is surprising because it contradicts Pyke's (1978) reasoning that bees visit high-rewarding flowers first. If Pyke was right, there would have been a penalty (negative *D*, resulting in strong geitonogamy) for a high nectar reward in male flowers of *Echium*. Apparently, this is not the case. The strategy of the plant is to combine protandry with high nectar production in the male flowers. This induces more visits to male flowers, but appar-

ently without bees visiting male flowers first. The advantage of protandry over protogyny in *Echium* remains, however, illusive. Bertin and Newman (1993) noted that in general protandry/protogyny is phylogenetically a rather conserved character. The pattern in the Boraginaceae, however, is variable and quite interesting. *Echium* spp., *Borago officinalis*, and *Phacelia* spp. are all protandrous and in all species the corolla opens widely. Bees typically crash into *Echium vulgare* flowers, like a baseball into a baseball glove. This effect is quite strong in *E. vulgare*. In the experiment reported by Rademaker *et al.* (1997), we began with bees visiting sequences of 2-day-old flowers from which we had removed all stamens. We stopped this procedure when we found that there was never any pollen deposited on the stigma. Bees slipped past the long style protruding from the corolla without pollinating the flower. Because of this effect a hypothetical long-styled protogynous *Echium* flower would likely have a problem receiving any pollen on its stigma. Perhaps this explanation, that after all pollen has been removed the remaining stamens assist and direct pollinator behaviour, extends to species with widely open corollas, other than *E. vulgare*. Borages with smaller tubular flowers such as *Pulmonaria officinalis*, *Symphytum officinale*, and *S. tuberosum* are adichogamous (ecoflora.co.uk), while *Cynoglossum officinale* is weakly protogynous. It would be interesting to examine whether this distinction applies to other borages and to other plant families with non-vertical inflorescences.

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