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THE DISTINCTION BETWEEN PREFERENCE
AND RANK ORDER IN POLLINATOR**

By

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

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Abstract [6-12—2010]

We develop a measure for quantifying rank order of visitation in complex sequences of male-phase versus female-phase flowers. The measure shows whether female flowers are visited before male flowers which enhances plant fitness. We apply the new method to bumble bee visitation in *Digitalis purpurea* and *Echium vulgare* and discuss our results in relation to the evolution of protandry in insect pollinated plant species.

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 the other way around, 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 are in fact self-incompatible (SI) (Lloyd &

Webb 1983, Bertin & Newman 1993). Apart from lowering the selfing rate, dichogamy can also increase seed set by reducing interference between pollen and pistils, even in species with 100% outcrossing. In addition dichogamy may increase pollen export to other plants (Darwin 1862 page 114; Bertin & Newman 1993; Routley & Husband 2006). These latter two consequences of dichogamy can apply to SI plants. The fraction of pollen lost from the bee when it forages between different flowers can be considerable since it not only involves pollen deposition on the stigma but also includes pollen falling on the ground and pollen groomed by the bee (Rademaker et al. 1997). Routley et al. (2004) found that protandry was associated with self-incompatibility, which fits in with the idea of extra consequences of dichogamy, in addition to reducing the selfing rate.

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 low, 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 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 in great majority protogynous (Bertin & Newman 1993) the opposite pattern occurs among animal-pollinated hermaphrodites. Intrafloral protandry is more common than protogyny (865 vs. 437 species and occurs especially among species with vertical inflorescences visited by bees (Bertin & 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, page 114) emphasized that *Spiranthes* is well adapted through a combination of (i) an acropetalous, vertical inflorescence in which flowers at the bottom open before those at the top, (ii) protandrous flowers and (iii) 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. This would also occur if the plant was flowering from top to bottom to (basipetalous) and flowers were protogynous. One would then expect species to have one of these favourable combinations; an acropetalous inflorescence coupled to protandry or a basipetalous inflorescence and protogyny. However, while the first combination occurs frequently, no species with the latter set of characters has so far been reported. McKone et al. (1995) found that *Besseya bullii* combined acropetalous flowering with protogyny. Lloyd & Webb (1986) mentioned *Cynoglossum officinale* as a possible example but this is incorrect since all boraginaceous plants flowers 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.

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

An important related aspect is nectar production, how this differs between the male and female phase and whether it has any effect in guiding pollinators. There is quite 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. He found that, after bagging flowers for 24 hours, 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 Byrzychudeck (1982) worked along similar lines, bagging flowers on *Digitalis purpurea* plants for 24h. They also observed a gradient from high nectar reward at the bottom to low reward in the top. Charnov (1982, p.259) concluded from Pyke's data that this suit 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 this addition. Indeed many pollinators like bees and hawkmoths typically forage from bottom to top on the inflorescence, but it is not so

clear that they visit the high rewarding flowers on the gradient first and then leave when rewards become too low. Without going into detail Lloyd & Webb (1986) already commented: “The upward movements of pollinators are often described as optimal foraging strategies, but the direction of movement does not always correspond to the postulated gradient of rewards”. Lloyd & Webb (1983) further outlined that there are obvious reasons for pollinators to forage from bottom to top; these reasons include minimising foraging costs, better flight control, better view and reducing predation. Darwin (1862) wrote: “... it is the most convenient method; on the same principle as a woodpecker always climbs up a tree”. One may also question whether the nectar production rate (NPR) is really high at the bottom and low in the top as Pyke (1978) and Best and Bierzychudek (1982) claimed. These authors used plants that were bagged for 24h. 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 24h after bagging does prove that female flowers have a higher NPR and higher nectar standing crop under open pollination conditions with bees continuously visiting the flowers. Contrary to the claim of high nectar production in female flowers, many studies found highest NPR in male flowers (Carlson 2007 and references therein). This is in line with Bell’s (1985) principle that in general more visits per flower are needed for pollen removal than for fertilization. When male flowers are associated with high nectar, then according to Pyke (1978) optimal foraging pollinators would be inclined to visit the high-rewarding 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 would be non-adaptive for the plant. So the question is how nectar production is related to Darwin's syndrome and how it affects pollinator movement?

All species mentioned until now have vertical inflorescences with old flowers at the bottom and new flowers at the top and they all fit Darwin's syndrome (further examples like *Gladiolus* and *Epilobium angustifolium* are mentioned in Lloyd and Webb 1986). However, some protandrous species flower along subbranches, i.e. non-vertical inflorescences, with young male flowers more peripheral than old female flowers. In such species, all Boraginaceae flower along non-vertical cymes, there is 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 affected by award?

We will argue that pollinators do not visit the most rewarding flowers first, but rather forage in a "convenient" way from bottom to top. We will make a distinction between (i) the preference that pollinators have for male-stage vs. female stage flowers and (ii) 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 both show the same preference for female flowers but differ in the rank order of visitation.

In this paper we develop a simple measure that allows quantifying rank order for any foraging bout including male and female flowers. When pollinators always forage from bottom to top and old flowers are always at the bottom and new ones at

the top, then observations like Darwin made suffice. However, when the inflorescence is less well structured and bees also made apart from upward movements also lateral or even downward movements, we need some quantitative measure of whether bees visit female flowers before male ones. One still expects that protandry is adaptive when female flowers are visited, on average, before male flowers. One way of quantifying rank-order of visitation is to distinguish between male-female and female-male transitions (McKone et al. 1995). For protandry to be favourable one expects that female-male transitions to be more common 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 certainly 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 on bumble bee foraging on *Digitalis purpurea* and *Echium vulgare*, both species with markedly protandrous flowers. As noted above, the *D. purpurea* inflorescence has a clear structure with old female flowers at the bottom of the vertical inflorescence and young male flowers in the top. In *E. vulgare* flowers open along cymes, i.e. from the inside of the plant to the outside. In *E. vulgare* there is no clear vertical gradient in the age of flowers or the associated nectar production.

Method

Potted flowering plants of *Digitalis purpurea* and *Echium vulgare* were placed in the field between 10 and 17 June 2010. Most bumble bees 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 could therefore let themselves drop after reaching the top of 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 µl micro capillaries. We measured nectar content in open flowers with continuous visitation. We also placed plants in a netted cage on 17.00h and measured nectar on 9.00h on the following day. Observations on bout length were made on four days for each species and we used only bout lengths of 6 flowers or more in the analysis.

For *Echium vulgare* no nectar was measured. However, Corbet (1978) already showed that NPR is higher in young male flowers and Rademaker et al. (1999) found a strong preference of bumble bees for these male flowers. Flower colour changes from pink to blue in *Echium vulgare*. Bees may therefore use color as a cue. Several observations on *E. vulgare* suggested that bees can assess nectar before entering the flower. Bees briefly halt before entering a flower or passing on to the next one (Rademaker & Taal 1998). Bumblebees will not enter a young flower filled with (scentless) sugar water. After adding nectar to old fumbled, female flowers that bees would normally skip, bees were observed trying to open the flower and access the nectar (de Jong personal observations).

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 in which 0 denotes again 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 $A_m=(3+6)/2=4.5$. If the difference between A_m and A_f gives a positive value, this shows that the bee visits the female flowers first; a negative difference shows that the bee first goes to the male flowers. 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 ten flowers. In equation:

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

Both flower types need to be visited before D can be calculated; so all male or all female sequences are removed from the analysis. D always varies between -5 and +5. For instance, extremes in bout length of $n=10$, like 0000000001 or 0000000011 or 000000111, all result in an average rank difference of 5 and therefore $D=5$. Because of the scaling this also applies to shorter (for example, 01 or 0011) or longer bouts in which all female flowers are visited before the male flower; in all instances $D=5$. For symmetrical sequences like 1001 or 001100, $A_m=A_f$ and D is always zero. By generating random visitation sequences we have convinced ourselves that the average of D is zero, when there is no preference of the pollinator to visit male or female flowers first. From analysing random visitation sequences we also found that the distribution of D is symmetrical around zero and fits well to a normal distribution when $n>10$. This is even the case when the probability p of visiting a male flower is

smaller or bigger than 0.5 in a setup with one male and one female flower. In such cases in the calculation of D sequences of only 0's and 1's need to be skipped. When $p > 0.5$ more male only sequences than female only sequences are taken out of the calculation. While this would affect average visits per male or female flower, it did affect D in many calculations we made using random numbers.

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 higher than in male flowers (0.65). The latter difference was not significant (Mann-Whitney test $P=0.07$ $n_1=15$, $n_2=17$). Averaged over the whole dataset female flowers received c. 15 % fewer visits per flower than male flowers.

We analyzed 52 bee approaches over the whole observation period of four days. D was on average 1.97 with a 95%-CL of 0.79 and therefore significantly bigger than one. Bees tend to visit female flowers before visiting male flowers. As noted by previous authors (Best & Bierzychudek 1984) the foraging pattern of the bees on *Digitalis* is typically from bottom to top.

For *E. vulgare* on all days bees greatly preferred visiting male flowers to female flowers. Overall a male flower received 3.6 times more visits than a female flower and the pattern was consistent over the three observation days (3.91, 3.58 and 3.27). 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. On the second and third observation day this trend was not observed. D was not significantly different from zero, i.e. bees visit 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 from Best & Biezychudeck (1984) that bees move from bottom to top of the plant and bees therefore visit female flowers first and visit male flowers before leaving. In our case however bees go against the nectar gradient, i.e. they begin with the lowest rewarding female flowers at the bottom and finish with the most rewarding male flowers in the top. The *Digitalis* results that protandry has the benefits sketched in the introduction. Note however that the average rank difference in rank between male flowers and female flowers is only *c.* 2, which is far from the ideal pattern for the plant when bees always visit female flowers first ($D=5$). If we had used plants *Digitalis* 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, in *E. vulgare* there was on day 1 a small (1.3 rank difference in a bout length of 10) tendency for female flowers to be visited before the male flowers. This may reflect the tendency of bee to visit cymes from inside out. On the other two days no difference in rank of visitation occurred. The result is unsurprising when we accept that bees forage from bottom to top. On the other hand the result is surprising because it fully contradicts Pyke's (1978) argument that bees visit high awarding flowers first and leave the plant when the yield per

flower has become to low. If Pyke were right there would have been a penalty (negative D , resulting in strong geitonogamy) on high nectar 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, apparently without bees going for these flowers first. The advantage of protandry over protogyny in *Echium* is however still illusive. Bertin & Newman (1993) noted that in general protandry/protogyny is phylogenetically a rather conserved character. Nevertheless the pattern in the Boraginaceae is variable and quite interesting. In *Echium* spp., *Borago officinalis* and *Phacelia* spp. the corolla is widely opened. Bees typically crash into *Echium vulgare* flowers, as a ball in a baseball glove. After removal of the anthers, bees typically go past the long style without pollinating the flower. This effect is quite strong in *E. vulgare*. In the experiment reported in Rademaker et al. (1997) we started with bees visiting sequences of emasculated flowers (whole anthers removed) and stopped this procedure when we found that in this way there was never any pollen deposited on the stigma. Due to this effect a hypothetical protogynous *Echium* flower would probably have a problem receiving any pollen on its stigma. Perhaps this explanation for *Echium* extends to species with widely open corollas. Borages with smaller tubular flowers like *Pulmonaria officinalis*, *Symphytum officinale* or *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. As Lloyd & Webb (1986) noted the problem of the evolution of protandry has hardly been addressed for these species with non-vertical inflorescences.

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

Plant No.	Day 1	Day 2	Day 3
1	0.89 ¹	-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(SE)	1.33(0.35)	0.23(0.23)	0.12 (0.26)

¹ Values present means of various (between 2 and 13) bout lengths on the same plant

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