



Differential pollination success in the course of individual flower development and flowering time in *Gentiana pneumonanthe* L. (Gentianaceae)

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The effect of flowering time and anthesis stage on fecundity of *Gentiana pneumonanthe* L. (Gentianaceae) in a greenhouse experiment was studied. The species is self-compatible, not apomictic, and rarely self-pollinates spontaneously. On average, flowers lived for 11.4 days, starting with a male stage of 3.6 and continuing with a female stage of 7.9 days. Pollen viability was highest during the first two days of the male stage, but never exceeded 50%. Seed-set from cross-pollination was higher earlier in the flowering period (77%) than later (63%). For self-pollinations, no significant difference was observed. The success of hand-pollinations declined dramatically after the fourth day of the female stage. Self-pollination yielded lower seed-set on the first day of the female stage than on the following three days. Also, there was a significant difference in seed-set between self- and cross-pollinations (25% vs 55%, respectively) only on the first day. This suggests that selection against self pollen was stronger during the first stage of the female phase and weakened as the stylar tissue aged. In conclusion, two different ways to enhance outcrossing were observed: (1) within the population's flowering period, and (2) within an individual flower's lifespan. Both may have important effects on the genetic variability of *G. pneumonanthe* offspring under unfavourable conditions. These include small population size (associated with reduced pollinator service), general pollinator scarcity, or rainy summers.

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ADDITIONAL KEYWORDS: Fecundity – floral longevity – pollen viability – reproductive success – seed-set – sexual selection – stigma receptivity.

INTRODUCTION

Pollen longevity and stigma receptivity are among the best-studied factors known to affect floral fecundity. Pollen viability is known to decline, sometimes rapidly, with age and exposure to environmental stress (Johri & Vasil, 1961; Nepi & Pacini, 1993; Thomson *et al.*, 1994; Aizen & Rovere, 1995). In general, plants with entomophilous pollination have pollen with longer viability than those of anemophilous species (Nepi &

Pacini, 1993). In particular, grasses may have a very short pollen viability of 30 min or less (Shivanna & Johri, 1985). Reduced pollen viability may result in a pollen limitation of seed-set, which is very commonly observed in nature. Reduced seed-set after seemingly adequate (experimental) pollination has been ascribed to sexual or gametophytic selection, female choice, nonrandom fertilization, etc. (Galen, Shykoff & Plowright, 1986). However, only rarely has pollen viability been investigated at the same time. This implies that, when studying mating systems in plants, we need to assess pollen viability to make sure that we do not obtain biased results (Thomson *et al.*, 1994; Stone,

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Thomson & Dent-Acosta, 1995). On the other hand, for effective pollination, not only must pollen be transferred to the stigma, it must also be deposited during its receptive period. After reviewing *c.* 1000 plant species, Heslop-Harrison & Shivanna (1977) found that the type of receptive stigmatic surface is associated with the self-incompatibility system of the species.

Both pollen viability and stigma receptivity are related to floral longevity, which may be either endogenous (i.e. time-dependent) or exogenous (i.e. pollination-induced). Pollination-induced senescence may have important consequences for the pollination system, and ultimately for a plant's reproductive strategy. For instance, in hermaphrodite flowers, senescence may limit the duration of pollen and stigma presentation and there will be strong trade-offs in any dichogamy system (Lloyd & Webb, 1986; Webb & Littleton, 1987). On the other hand, changes in pollen germination rate (Cruden, Hermannutz & Shuttleworth, 1984) and pollen-capturing efficiency (Smith-Huerta & Vasek, 1984) during anthesis have been seen as mechanisms that promote outcrossing and reduce deleterious effects of foreign pollen receipt.

The aim of this study was to investigate the extent to which pollen viability and stigma receptivity are time-dependent, and whether fecundity changes during self and cross-pollination both within anthesis and within the flowering period. We performed our experiments on *Gentiana pneumonanthe* L. (Gentianaceae), a perennial species of wet heathlands and hay meadows of temperate Europe. The species is increasingly rare in The Netherlands and legally protected (Mennema, Quené-Boterenbrood & Plate, 1985). Factors affecting population viability have been thoroughly studied by Oostermeijer (1996). In its natural environment, the flowers of this species are often not visited by any insect for prolonged periods, owing to bad weather conditions, small population size and a general decrease in species diversity of its habitat (Oostermeijer *et al.*, 1998). At the same time, the strict dichogamy of the species, as well as the fact that senescence of *G. pneumonanthe* flowers is pollination-dependent, made this species very interesting to study both from theoretical and applied viewpoints.

All experiments were carried out under controlled conditions in the greenhouse. This meant that fruit and seed development were not (or less) subject to drought, and competition for light and nutrients as compared to field conditions (Petanidou *et al.*, 1995a). In addition to our main goal of investigating whether fecundity characteristics of *G. pneumonanthe* are time-dependent, we also studied the effects of different pollination treatments under greenhouse conditions. We compared the results of this experiment with those obtained in a similar study carried out in the field (Petanidou *et al.*, 1995a).

MATERIAL AND METHODS

The study species

Gentiana pneumonanthe (Gentianaceae), the marsh gentian, is a herbaceous perennial plant that reaches 50 cm. An adult plant bears 1 to 25 flowers, a number that may vary from year to year. The species occurs only in wet heathlands and hay meadows of temperate Europe from the northern Balkans to southern Scandinavia. Closely related species extend into temperate Central Asia. In The Netherlands, *G. pneumonanthe* used to be far more widespread before 1950 than it is today (Mennema *et al.*, 1985). The reduction concerns both the number of localities and the average population size (Oostermeijer, 1996). The decline is mainly a result of a loss of suitable habitats and changes in (agricultural) land use. Therefore, the only sites where *G. pneumonanthe* still occurs in The Netherlands are nature reserves in which wet heathlands and nutrient-poor, unfertilized, moist hay meadows are maintained by means of sod-cutting, mowing or grazing (Oostermeijer *et al.*, 1992; Oostermeijer, van 't Veer & den Nijs, 1994a; Oostermeijer, 1996).

For the purpose of our study, we used more than 200 potted individuals. The plants were raised in the greenhouse from seeds. All experiments have been carried out at the University of Amsterdam under greenhouse conditions in summer 1989, except for the pollen viability experiment, which was carried out in the greenhouse at the Catholic University of Leuven (KUL) on potted plants from the same collection in summer 1994.

Flower characteristics

Flower development and lifespan were investigated from 5 to 30 June 1989 on 11 flowers, each on a separate individual. The flowers were marked in the bud stage, and pollen and stigma presentation were monitored once a day until the flowers withered. The timespan of the different anthesis stages was expressed in full days.

In order to determine the number of pollen grains produced per flower, a number of fresh flowers ($N=7$) with undehisced anthers was sampled at random. The five anthers of each flower were mixed with one tablet of a known number of *Lycopodium* spores ($N=13500$), acetolysed and processed according to Faegri & Iversen (1975). The total number of pollen grains per flower was estimated from the ratio of *Gentiana* pollen grains to *Lycopodium* spores counted in the mixture.

Pollen viability and stigma receptivity were measured on 13 flowers, each on a separate, newly flowering plant from 15 September to 7 October 1994 in the greenhouse of the Botanical Institute of the KUL. The anthesis of these flowers lasted 5 to 9 days.

Pollen viability was assessed through a germinability test. We used seven flowers which we sampled for pollen directly from the anther once per day (at 10:00 am). Each studied flower was sampled separately from its first day of opening until it withered. The daily sampled pollen from each flower was used to prepare six germination samples (seven flowers \times six samples) using the Brewbaker–Kwack medium (Kearns & Inouye, 1993). After preliminary tests with 5–60% sucrose solutions, we found that a 10% sucrose solution was the most appropriate. Each pollen sample (± 200 grains) was sprinkled and mixed well with a droplet of the Brewbaker–Kwack medium under a dissecting microscope. The droplets (= samples) were left hanging from the lid of a Petri dish at room temperature. After 24 h, germination was stopped by freezing (Dafni, 1992). Each sample was transferred onto a slide, permanently mounted and examined under a phase contrast microscope (1–2 samples per flower and per day, selected at random). Only the pollen grains that successfully germinated and produced visible tubes were considered viable. Grains which had produced blister-like protrusions or other uncertain cases were classified as not germinated. Pollen viability was expressed as the percentage of germinated pollen grains.

We estimated stigma receptivity with the benzidine- H_2O_2 method as described by Dafni (1992). Twenty-one flowers were used, three for each female age class (first to seventh day of the female stage). When receptive, the entire stigma stains blue after immersion into the benzidine- H_2O_2 solution. It should be underlined that this measurement is a qualitative one, not showing the quantity of pollen that can germinate on the stigma, which is also related to the time it takes the stigma to unfold. Therefore, in order to evaluate floral fecundity at each day of the female stage we relied on an earlier experiment performed in 1989. This experiment is described in the following paragraph (second set of experiments).

Reproductive success after different pollination treatments

We carried out two sets of pollination treatments, the first set from 2 June to 15 July, the second from 23 July to 1 August 1989.

The first set of experimental treatments aimed at investigating the reproductive success of the species under greenhouse conditions. Experiments to study self-compatibility and the success of spontaneous self-pollination were carried out on the same individuals, each covered by a metal cage with fine gauze to prevent the access of any insect that might have entered the greenhouse. Flowers were marked in the bud stage on the calyx with acrylic paint. They were either manually

self-pollinated during the female stage ($N=88$) or remained untouched ($N=102$). The success of cross-pollination was estimated on uncaged flowers that were marked with a different colour, and left uncovered until harvesting ($N=78$). All hand-pollinations were carried out on the fully exposed stigmas of the second day of the female stage and only once per flower, using an abundant quantity of pollen from a single flower donor. Manual selfing was conducted with the plants' own pollen, obtained as freshly as possible, usually from other flowers on the first or second day of the male stage. For the outcrossing treatment, we obtained fresh pollen from flowers on other plants, which were in the first or second day of their male stage as well. A check on possible apomixis was carried out on 47 flowers which were emasculated in the bud stage and caged. In order to be sure that no pollen germinated on the stigmas, the continuously unfolding stigmas were gradually removed until the end of anthesis.

The aim of the second set of experiments was twofold: (1) to detect whether the mating system of *G. pneumonanthe* was time-dependent, i.e. whether its reproductive success was related to the point of time within the population's flowering period; (2) to detect possible differential floral fecundity at different days of the female stage. The treatments were carried out on 242 flowers on c. 100 individuals. The buds were divided into two groups: the first ($N=125$) were manually self-pollinated and the second ($N=117$ on the same plants as the first group) were cross-pollinated by hand. Before treatment, each flower had been monitored since the time of opening, so the exact day of the female stage was known. Self-pollination (first group) was performed with pollen from freshly opened flowers of the same individual, and outcrossing (second group) with pollen coming from a freshly opened, male flower of a single other individual. Hence, we used only one pollen donor per cross-pollination treatment. Manual pollinations were carried out using an abundant quantity of pollen, and only once per flower. One or more anthers were brushed across the unfolded stigma until its surface was entirely covered with pollen. The flowers were marked with a different colour of acrylic paint for each group, caged, and left untouched until harvesting. We pollinated flowers on day 1, 2, 3, 4, 5 or 6 of the true female phase (see Results). In both groups, some of the followed flowers closed on the first or the second day of their female stage, before being manually pollinated. As the cause of this was unclear, we omitted these flowers from further analyses.

All treated flowers set fruits that were harvested c. 6 weeks after treatment. As a standard procedure, 30–40 fruits (first set of experiments in June) or 10 fruits (second set in July) for each treatment and each day of the female stage were selected at random from

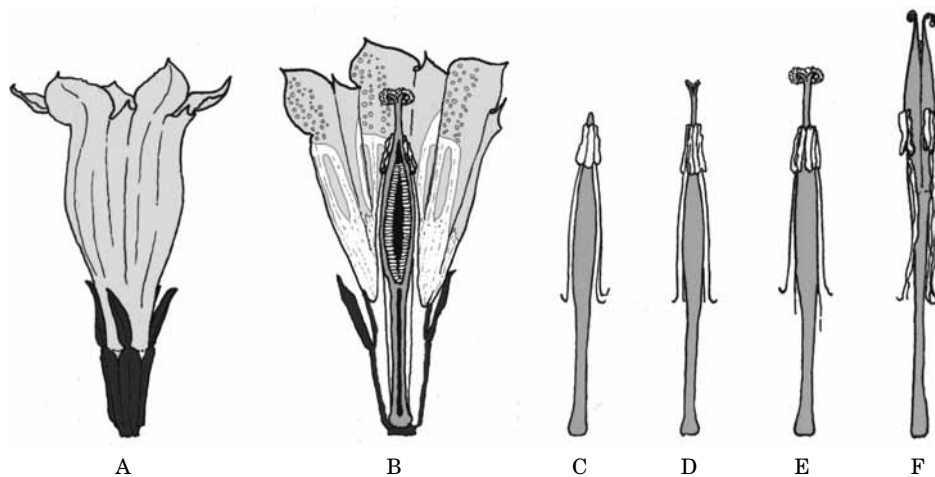


Figure 1. Flower of *Gentiana pneumonanthe* in different stages: (A) outer side; (B) female phase in longitudinal cross-section, showing the nectar guide. Details: (C) male, (D) early female, (E) late female, (F) mature fruit on gynophore with withered stamens and stigma. Drawing: G. Oostermeijer.

the total number of the harvested fruits to evaluate reproductive success. We determined the latter by counting the number of filled (=viable) and empty, shrivelled (=aborted) seeds (cf. Petanidou *et al.*, 1995a). The sum of the viable and aborted seeds will be considered here as equal to the total initial number of ovules. Seed-set is the percentage of viable seeds over this initial number of ovules.

Statistical analysis

Prior to statistical analysis, the data were tested for normality using Kolmogorov–Smirnov tests (Sokal & Rohlf, 1995). Because in several cases the data were not normally distributed, and transformations did not improve this, we applied non-parametric tests. Variation in reproductive success as a result of the pollination treatments was tested by Kruskal–Wallis ANOVA followed by separate Mann–Whitney *U*-tests after application of the Bonferroni correction (Sokal & Rohlf, 1995). Throughout the paper, the mean values are followed by their standard error (SE).

RESULTS

Flower morphology and anthesis characteristics

Gentiana pneumonanthe bears actinomorphic, funnel-type flowers. The inner tube is greenish blue, and bears a nectar guide on each of its five facets. The guiding pattern consists of green and white stripes and light green dots and rings, and is clearly visible to the naked human eye (Fig. 1A–F). The corolla lobes are blue to mauve, and have appendages in the sinuses. All floral components are rather odourless, except for the nectar, which has a very distinct, sweet smell.

Detailed description of the floral pattern is available in classical anthecology handbooks (Sprengel, 1793; Knuth, 1899, and literature cited therein). Among them, the most informative is Kerner von Marilaun (1891), who interpreted (in the very similar *G. asclepiadea* L.) the folding and the continuous elongation of the corolla as a mechanism enhancing autogamy in the context of maximizing reproductive success.

Flowers were strongly protandrous, and had a life-span of 11.4 ± 1.25 days ($N=11$). Anthers dehiscid immediately after anthesis had initiated. The male phase lasted 3.6 ± 0.28 days, including the period of the style development (1.3 ± 0.20 days) (cf. Fig. 1C). The ‘true’ female phase of anthesis started from the beginning of the unfolding of the stigma and the exposure of its receptive papillae to pollen (Fig. 1D, E). This stage lasted 7.9 ± 1.06 days.

After manual pollination, the flowers responded by closing within a couple of hours. Thereafter they remained closed until they withered. This failure to reopen after pollination by hand was observed particularly early in the greenhouse flowering period. Later on, in July, some of the flowers closed less promptly after the treatment; they remained semi-closed, sometimes opening over a couple of days following hand-pollination.

Under greenhouse conditions, the flowering of *G. pneumonanthe* started in May, 2 months earlier than in nature. Under both conditions, flowering finished by the beginning of October (Petanidou *et al.*, 1995a).

Pollen viability and stigma receptivity

The average number of pollen grains per flower was $6.6 \times 10^5 \pm 1.6 \times 10^5$ ($N=7$). The results of pollen germination tests are illustrated in Figure 2. There was

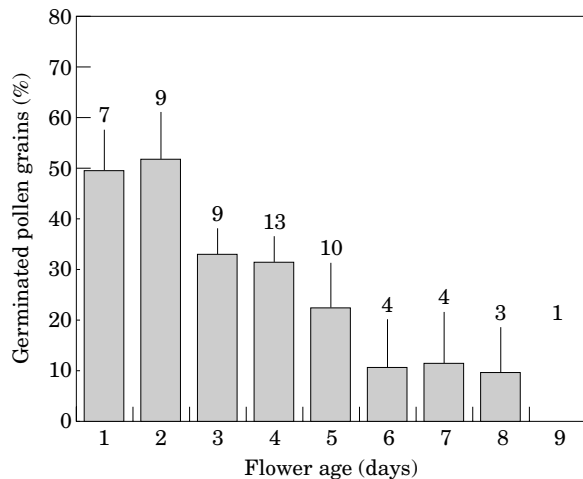


Figure 2. Pollen viability, expressed as percentage of germinated pollen grains in samples from flowers of *Gentiana pneumonanthe*. Error bars on the top of the columns represent the SE and the numbers the sample size (total number of slides, at least one per flower studied).

an obvious gradual decline in germination from c. 50% on the first two days of anthesis (range 89.3–16.4%) to zero on the ninth day.

The stigma was found to be receptive throughout the entire female stage (first to seventh day). Another assessment of receptivity, in purely quantitative terms, is given below.

Reproductive success and flowering time

All flowers produced ripe capsules, regardless of the type of treatment. Figure 3 shows the seed production and estimated initial number of ovules after the treatments in June and in July.

During the pollination experiments early in the flowering period (June), seed-set differed among treatments (K–W $H_{(3,147)} = 110.371$, $P < 0.0001$). Post-hoc tests showed that there was a highly significant difference between all treatments (M–W U -tests with Bonferroni correction; $P < 0.0001$). Similar results were obtained for seed numbers (K–W $H_{(3,147)} = 105.856$, $P < 0.0001$; M–W U -tests, $P \leq 0.001$). There was no difference between treatments in the number of ovules (K–W $H_{(3,147)} = 3.317$, $P = 0.345$).

Flowers that were subjected to the apomixis treatment in June produced virtually no seeds at all (seed-set $< 0.1\%$). The same was observed for the spontaneous selfing treatment (Fig. 3). Cross- and self-pollination treatments in July differed in the number of viable seeds ($U_{(30,30)} = 590.5$, $P = 0.038$) and marginally in seed-set ($U_{(30,30)} = 577.5$, $P = 0.059$). Again, there was no difference in the initial number of ovules ($U_{(30,30)} = 462.5$, $P = 0.853$).

The experiments early in the flowering season (June)

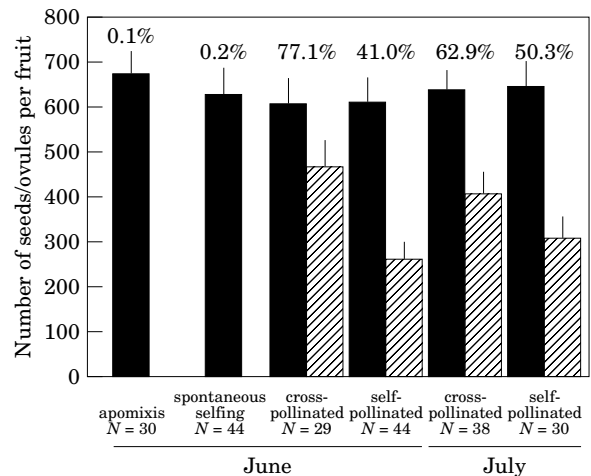


Figure 3. Reproductive success of *Gentiana pneumonanthe* after the treatments in June and in July. Columns represent the mean initial number of ovules (■; viable + aborted seeds) and the mean number of viable seeds (▨) per fruit. The percentage seed-set (back-transformed means) is given on top of each pair of columns. Error bars represent the standard error of the mean. The sample number is given in the base of the columns.

were compared with the results of pollinations performed later on, in July, on flowers that were in days 2–4 of the female stage (see above). Cross-pollination in the later experiment resulted in a lower seed-set than the early treatment ($U_{(29,30)} = 631$, $P = 0.003$) due to the slightly higher initial number of ovules produced per flower in July compared to June ($U_{(29,30)} = 307.5$, $P = 0.053$). Apart from this, we observed no other significant differences between the two periods ($P \geq 0.136$).

Reproductive success and flower age

Seed-set depended strongly on which day of the female stage the flower was pollinated. This was true after both manual selfing and outcrossing (Fig. 4). For both treatments, the pollination or fertilization success declined dramatically after the fourth day of female stage. Flowers that closed before having been pollinated practically set no seeds (2.0 ± 1.50 , $N = 14$). There was a difference in seed-set between selfed and outcrossed flowers, but only for those that were pollinated on the first day of the female stage (M–W $U_{(9,10)} = 71.0$, $P = 0.034$). For flowers that were pollinated on day 2, the difference in seed-set after selfing and outcrossing was only marginally significant ($U_{(9,10)} = 67.0$, $P = 0.072$). No differences were found on the third and fourth days ($P \geq 0.342$). The seed-set of selfed flowers was lower when pollinated on the first day than when pollinated on day 2 ($U_{(10,10)} = 78.0$, $P = 0.034$) and day 4 ($U_{(10,10)} = 82.0$, $P = 0.016$), but not on day 3 ($U_{(10,10)} = 66.5$, $P =$

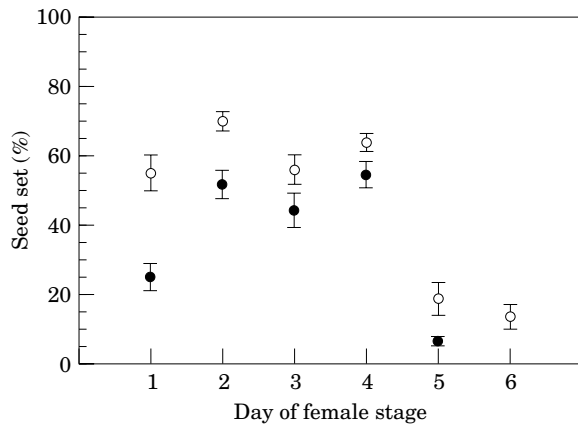


Figure 4. Seed-set (% \pm SE) obtained after cross-pollination (○) and self-pollination (●) on different days of the female stage.

0.212). These differences were not found among the outcrossed flowers ($P \geq 0.354$).

DISCUSSION

Parameters affecting the mating system

Gentiana pneumonanthe shows dichogamy (protandry, Lloyd & Webb, 1986) and approach herkogamy (Webb & Lloyd, 1986; Lloyd & Schoen, 1992), which are also found in other gentian species (Spira & Pollak, 1986; Webb & Pearson, 1993). Partly as a consequence of these syndromes, spontaneous self-pollination resulted in a very low seed-set, despite the rather high self-compatibility (Fig. 3).

We conclude that an animal pollen vector is indispensable for good reproduction, confirming Sprengel's (1793) prediction based on floral structure, dichogamy and the flower's sleeping habit. This holds for the entire flowering period, both indoors and outdoors (cf. Petanidou *et al.*, 1995a). In nature, *G. pneumonanthe*, like the other gentians, is pollinated by bumblebees, primarily by the workers of *Bombus pascuorum*, which visit the flowers for nectar (in The Netherlands; Petanidou *et al.*, 1991, 1995a,b, 1998). However, pollinator visitation probably depends on population size and the percentage cover of co-flowering species, such as the heather species *Erica tetralix* and *Calluna vulgaris*, because natural seed-set was positively correlated with both variables independently in a series of populations in The Netherlands (Oostermeijer *et al.*, 1998). Because pollinator availability and behaviour are highly variable in its natural habitat, we can infer that *G. pneumonanthe* most likely has a mixed mating system (Brown, 1990). In large populations, which are usually found in vegetation communities rich in flowering plant species, flowers probably receive a sufficient number of bumblebee

visits during the peak flowering season to ensure a high outcrossing rate. Later in the season, or in small populations in grass-dominated vegetation, flowers will be visited rarely, and spontaneous selfing will frequently occur. A positive relationship between population size and outcrossing rate has been observed in *G. pneumonanthe* (Raijmann *et al.*, 1994).

Changes in pollination success in the course of the flowering period

In the field, the success of spontaneous selfing was, although still rather low, much higher (25%, Petanidou *et al.*, 1995a) than we observed in the greenhouse (0.2%). The lifespan of flowers was much longer in the greenhouse (11.4 days) than in the field (3.9 days). Although it may seem that this increased the number of opportunities for pollen transport from anthers to stigma, e.g. because the flowers opened and closed more often, the strong decline in pollen viability after three or four days probably rules out the time advantage. There are several possible explanations for the failure of spontaneous pollination in the greenhouse. Firstly, there is much more movement of the flowers by wind under field conditions, so that transport of pollen within the flower is more likely. Secondly, seed-set after spontaneous selfing in the field also failed completely (seed-set 0%) under the adverse conditions of October. This would suggest that the conditions in the greenhouse were not really optimal for seed development. This hypothesis is supported by two additional observations: (1) in the field, pollinations during the peak season in August had higher seed-set (80–86%) than the pollinations performed early (during the 'peak') in the greenhouse (41–77%); (2) when pollinated in October in the field, seed-set of manually selfed flowers declined from 82 to 64%, a level that is more similar to that observed in the greenhouse (Petanidou *et al.*, 1995a).

It is strange that the seed-set of outcrossed flowers decreased from June to July in the greenhouse, whereas the seed-set of selfed flowers did not. In contrast to the selfed flowers, the plants used for the outcrossing treatment were both uncaged and emasculated. Especially later in the flowering period, when conditions apparently became less favourable, the sheltering offered by the cage and the absence of damage to the corolla from the emasculation may have resulted in a lower seed abortion in the selfed flowers.

Hence, on the basis of our experiments, we must conclude that pollination 1 month later in the flowering period had only a small effect on seed-set. Probably, 1 or 2 months extra delay in pollination would have had more significant effects, similar to those observed between August and October in our earlier field study (Petanidou *et al.*, 1995a).

Changes in pollination success in the course of flower development

The male reproductive success of a plant depends, in part, on the amount of pollen it donates to stigmas. In animal-pollinated plants, pollen transfer to other flowers is influenced by the schedules of pollen presentation, pollen survivorship, and pollinator visits (Thomson & Thomson, 1992). The number of pollen grains produced by a *G. pneumonanthe* flower is very high, and normally available from the first day of anthesis. However, the pollen was on average only c. 50% viable, and this holds only for the first two days of anthesis (Fig. 2). After the fourth or the fifth day, pollen germinability declined dramatically. Such a low pollen viability implies that for plants to be most fit under natural conditions, pollen uptake by visiting bumblebees should be accomplished as early as possible after the flower opening, preferably not later than the second day of anthesis. Since during the peak season, *Gentiana* flowers in a large population received an average of 3–4 visits each day (Petanidou *et al.*, 1995a), the probability is quite high that most pollen is removed when still fresh and maximally germinable. Because pollen that remains in the flower until the stigma becomes receptive can probably compete less successfully with the fresh pollen imported from flowers in the male stage, the sharp decline of pollen viability may be an adaptation to increase the level of outcrossing within a population.

On the other hand, the degree of pollen transfer also depends strongly on the frequency of rainy days, during which the functional flowers remain closed and unvisited. Our data show that when pollination is postponed (e.g. due to bad weather conditions), *G. pneumonanthe* may experience a sort of pollen limitation within flowers due to the declining pollen quality. Brantjes (1983) also mentioned weather-induced loss of pollen viability as one of the selective factors on male function of *Isotoma* (Campanulaceae). Considering that bad weather also affects the female component of fecundity (cf. below) we conclude that in particularly wet summers, the seed production of *G. pneumonanthe* may be considerably reduced. As selfing rates and, consequently, inbreeding would increase at the same time, it can be expected that the average offspring quality is lower in bad summers as well (Oostermeijer *et al.*, 1994b, 1995).

There was a remarkable difference between self and outcross pollen in their ability to sire seeds on the first two days of the female stage. On subsequent days (3–6) of stigma receptivity, however, siring success of self and cross pollen was more or less equal. The results could have been caused by a variety of factors, such as differences in pollen tube growth rate, interactions between pollen and the stigma or style, position of ovules within the ovary, abortion of inbred embryos

through expression of lethal recessive alleles, or competition between embryos for resources (de Nettancourt, 1977; Marshall & Folsom, 1992; Rigney, 1995). An increasing rate of fertilization by self pollen tubes with flower age agrees with the observation that physiological interactions between pollen tubes and the stigma or style weaken as the female organs grow older (de Nettancourt, 1977; Haber & Frankie, 1982). This weakening of selection against self pollen would allow a certain degree of 'delayed selfing' to occur when the opportunities for outcrossing have passed, as has been predicted by Lloyd (1979).

In conclusion, we have shown several new aspects to the pollination biology of *Gentiana pneumonanthe*. In contrast to the apparently full self-compatibility we observed in the field, experiments in the greenhouse demonstrated that plants are, to some degree, able to increase the possibilities for outcrossing, probably by means of inhibiting the siring success of self pollen in the first days of the female stage. At the same time, the already low viability of pollen decreases rapidly over time, so that any self pollen remaining in the flower after the male stage has limited germination success on the flower's own stigma. In populations with sufficient visitation by bumblebees, and under good weather conditions, the mechanism observed will enhance outcross-pollination. However, since pollination limitation probably occurs rather frequently, especially in the fragmented and deteriorated landscape of today (Oostermeijer *et al.*, 1998), selfing may easily prevail over outcrossing and threaten the viability of the population by inbreeding depression (Oostermeijer *et al.*, 1994a,b; Oostermeijer, 1996).

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