

Pollination ecology of *Gentianella uliginosa*, a rare annual of the Dutch coastal dunes

T. Petanidou, A. C. Ellis-Adam, J. C. M. den Nijs and J. G. B. Oostermeijer

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The pollination ecology of *Gentianella uliginosa*, a rare annual in The Netherlands, was studied in three relatively small coastal dune populations. In all populations, the species was fully self-compatible: manual self pollination resulted in a seed set of 92-94%. Autofertility was also high (seed set of caged, unhandled flowers 80-85%). The homogamous flowers were visited by *Bombus pascuorum*, which collected nectar and transported pollen sternotribically. Differences among populations in bumblebee visitation rate did not affect natural seed set. In one plot, seed set following natural pollination was lower than after manual selfing, and as high as after spontaneous selfing. As flowers here were also visited frequently, this might be due to pollen limitation through heterospecific pollen competition. Support for this hypothesis was derived from another population, where seed set after natural pollination was lower in a plot dominated by co-flowering *Mentha aquatica* than in a plot with few *Mentha*. However, differences in water availability might also have caused this. In one population, seed sets of the cross pollination and spontaneous selfing treatments were lower than those of the manual selfing and natural pollination treatments. Whether the low outcrossing success can be attributed to small differences in the treatment of the experimental plants or to outcrossing depression remains unclear. We conclude that this annual species is most likely a predominant selfer. Its high autofertility is probably an adaptation to its wet dune slack habitat, where reproductive assurance may be important to cope with unpredictable fluctuations in water table, summer droughts and pollinator service.

T. Petanidou, Dept of Geography, University of the Aegean, Faonos & H. Trikoupi, GR-8100 Mytilene, Greece. – A. C. Ellis-Adam, Institute for Systematics & Ecology, Hugo de Vries Laboratory, University of Amsterdam, Kruislaan 318, NL-1098 SM Amsterdam, The Netherlands. – J. C. M. den Nijs, Institute for Systematics & Ecology, Hugo de Vries Laboratory, University of Amsterdam, Kruislaan 318, NL-1098 SM Amsterdam, The Netherlands. – J. G. B. Oostermeijer, Institute for Systematics & Ecology, Hugo de Vries Laboratory, University of Amsterdam, Kruislaan 318, NL-1098 SM Amsterdam, The Netherlands.

Introduction

Due to the increasing fragmentation of their natural habitats, many plant species presently have smaller and more isolated populations than they were used to. Com-

puter simulations with data from natural populations have shown that small populations may experience greater risks of extinction in a changing environment than large ones (Menges 1991, Ouborg 1993, Oostermeijer 1996a). Several studies have demonstrated that

the genetic structure of small populations is influenced by genetic drift, which results in loss of genetic variation (Barrett & Kohn 1991, Ouborg et al. 1991, van Treuren et al. 1991, Ellstrand & Elam 1993, Ouborg 1993, Widén & Andersson 1993, Rajmann et al. 1994, but see Lewin 1987). Small populations may also exhibit inbreeding through higher selfing rates and an increased incidence of mating between close relatives. In plants with a mixed mating system, reduced population size may negatively affect the balance between selfing and outcrossing (Templeton 1986, Charlesworth & Charlesworth 1987, Oostermeijer et al. 1992). In animal-pollinated, predominantly outbreeding plant species, reduced population size may also have direct effects on seed production (Jennersten 1988, Kwak 1988, Kwak et al. 1991, Petanidou et al. 1991, Oostermeijer et al. 1998). For instance, rare plants in small populations may become increasingly dependent on the presence of simultaneously flowering species, because they are unable to attract sufficient numbers of pollinators themselves (Kwak 1988, Petanidou et al. 1995a, Oostermeijer et al. 1998). On the other hand, small populations may also suffer from the presence of such neighbours, due to either competition for pollinators (Kwak & Jennersten 1991) or heterospecific pollen transfer (Thomson et al. 1981, Rathcke 1983, Waser 1983, Campbell & Motten 1985, Petanidou et al. 1995a),

In a project aiming to investigate the constraints and risks faced by small populations of rare species, we previously studied the reproductive biology of two perennial species of the genus *Gentiana* (viz., *Gentiana pneumonanthe* L. and *G. cruciata* L.; Petanidou et al. 1991, Oostermeijer et al. 1992, 1994a, 1994b, 1998, Petanidou et al. 1995a, 1995b). We found that although both species are fully self-compatible, pollinator visits were indispensable for their reproduction due to strong protandry and herkogamy. Moreover, seed set of *G. pneumonanthe* appeared to be dependent on the vegetation that surrounds the individual plant either because of differences in nutrient availability or facilitation of pollination through the presence of other insect-pollinated plant species (Petanidou et al. 1995a, Oostermeijer et al. 1998). Also, seed set of this species was positively correlated with population size (Oostermeijer et al. 1992, 1998), and there is evidence that inbreeding reduced offspring fitness (Oostermeijer et al. 1994a).

The annual *Gentianella uliginosa* is a rare and threatened coastal species in The Netherlands. Predictions about vulnerability of remnant populations are not necessarily the same for this annual species as for the two perennials we studied previously, since annuals and perennials are likely to differ in reproductive strategy (Levin 1972, Lloyd 1979, 1992). The aim of this study on *G. uliginosa* was to investigate (1) the effects of dif-

ferent modes of pollination on potential seed production and realized seed set, (2) the dependence of the species upon the local pollinating fauna, and (3) the possible effects of co-flowering species on reproductive success.

Materials and methods

Study material

The taxonomic position of the plant material of the populations studied proved to be problematic. According to the recent Dutch floras (Mennema 1994, van der Meijden 1996), *Gentianella amarella* (L.) Börner is the only species of the *amarella*-group occurring in The Netherlands. However, according to the Flora Europaea (Pritchard & Tutin 1972) only *G. uliginosa* (Willd.) Börner, is present in this region. After a survey of a limited sample of herbarium material from diverse European origins kept in AMD, we conclude that most likely our study plants belong to a single, phenotypically highly variable taxon. Most individuals, however, fall within the range of *uliginosa*. We consider it beyond the scope of this study to formalize this conclusion as this would require a full taxonomic revision, and we limit ourselves momentarily to pointing to this problem. In this paper we will use the name *G. uliginosa*.

Our study populations consist of annual plants varying in height from 1 to 35 cm. Each individual may bear from 1 to 100 pinkish to dark purple flowers. The corolla tube is rather narrow, and up to 2 cm long. At the throat of the flower, there is a characteristic rim of fringes which vary in position: upright in sunny weather when the flower is fully open, whereas they close over the floral tube under dull weather conditions (Petanidou et al., unpublished data). On the basis of these observations, the functional role of the fringes may be discussed. By closing the entrance of the flower, they either protect the flower from small nectar-stealing insects (Kerner von Marilaun 1876), or prevent the dilution of the nectar by rain (Knuth 1899). The nectar, secreted from nectaries at the internal side of the corolla base between the insertion of the filaments, is accumulated around the base of the ovary.

Gentianella uliginosa (Willd.) Börner has a N and NC European distribution, occurring in wet to moist dune slacks and exposed coastal meadows (Pritchard & Tutin 1972). In The Netherlands, it occupies a rather narrow belt, which fluctuates in elevation from year to year in response to changes in the depth of the ground water. In dry years, plants are found at the bottom of the slack, whereas in wet years, they may grow higher on the slopes of the dunes. Owing to this, variation in annual population size is much higher than in both perennial gentians we studied earlier.

Study sites

Field experiments and observations were carried out during the summer of 1989. Two study sites were selected in the dune areas of the western coast of The Netherlands.

The first site is situated in the "National Park Zuid Kennemerland", 5 km W of Haarlem. Here, we studied two subpopulations, separated by 0.2 km and situated in a dune slack approximately 0.5 km behind the outermost dune ridge ("Kennemerduinen-1" and "Kennemerduinen-2", or K-1 and K-2). Each subpopulation occupied a patch of only a few hundreds of square meters. At the time of the study, the number of individuals was estimated at several thousands and ca. two hundreds of flowering individuals, respectively. Population K-1 exhibits more yearly fluctuations in size due to shifting sand and changes of the ground water table (den Nijs & Oostermeijer, personal observations). Population K-1 was surrounded by very few co-flowering individual plant species. This contrasted with population K-2, where the main co-flowering species were, in order of relative abundance: *Pulicaria dysenterica*, *Lythrum salicaria*, *Lotus corniculatus* subsp. *corniculatus*, *Euphrasia stricta*, *Prunella vulgaris*, and *Mentha aquatica*. Observations on pollinator behaviour were carried out in a plot of 3×3 m placed in the center of K-2. Reproductive potential and success after several pollination treatments (see below) was studied in both sites. The fieldwork was carried out during the local flowering peak (i.e., when almost all individuals had open flowers) for seven consecutive days from 14 to 20 August.

The second field site is situated in a nature reserve near Oostvoorne, c. 20 km WSW of Rotterdam. It comprises a single, continuous population ("Oostvoorne" or O). During the fieldwork, the population was estimated to consist of c. 2 000 individuals on approximately 0.4 ha. Here, plants were smaller in size than those studied in K-2. The dominant co-flowering species was *Mentha aquatica*, and to a lesser extent, *Prunella vulgaris*. The fieldwork was conducted during the peak of the local flowering period, from 3 to 8 September. Two plots of 3×2.5 m each, separated by 5 m (O-1 and O-2) were selected to study pollinator behaviour and pollination success. O-1 contained 334 flowers of *Gentianella*, and 99 flower heads of *M. aquatica*, and O-2 was dominated by *M. aquatica* (422 heads) and comprised 201 flowers of *Gentianella*.

Flowering characteristics and nectar standing crop

Flower life span was measured on flowers borne on different plants and positions within the plants; the flowers were marked in bud stage, and individually followed two or three times per day, until they withered. Only whole days were scored. The numbers of flowers followed were 31 in K-2 and 49 in O (plot O-1 and O-2 together).

The nectar standing crop of the flowers was measured only in the K-2 and O-1 plots. Measurements of the nectar volume were made by inserting 0.25 to 1- μ l microcapillaries (made by Drummond) into the corolla tubes to collect the nectar from their bottoms. The nectar was harvested from a number of open, uncaged flowers selected at random at different times of the day.

Flower visitors

The behaviour of the visiting insects was studied in K-2 (16 and 19 August) and O (4-5 September, in both O-1 and O-2 plots in parallel). During several time intervals, spread out over an entire observation day, a plot was monitored for 15 minutes by a single observer. In population O, another person simultaneously made similar observations in the second plot. We determined the numbers of bumblebees and other insect visitors in the plots, as well as the numbers of visits paid to *Gentianella uliginosa* and to the co-flowering species.

In order to check the interchange of pollen vectors between the two spatially separated Kennemerduinen subpopulations, 25 and 8 bumblebees were individually marked in K-1 and K-2, respectively (on 18 August), and released to be possibly recaptured in the other subpopulation during the following two days. We captured bees while visiting a *Gentianella* flower, anaesthetized them slightly with ethyl-acetate, and glued a coloured, waterproof number on their thorax. We marked only a small number of bumblebees in K-2 to minimize disturbance as we performed all observations on pollinator behaviour in that plot. This experiment also allowed us to assess whether the K-2 population was visited by different bumblebee individuals or by the same (or a few) individual(s) repeatedly.

The pollination efficiency of the bumblebees and of other visiting insects was assessed for the plots K-2, O-1, and O-2 by two different approaches. Firstly, the conspecificity and heterospecificity of the pollen loads borne on different parts of the insects' bodies was analysed. The loads were sampled from insects captured between two consecutive visits to the study species, inside or in the vicinity of the study plots. The bumblebees were lightly anaesthetized and the pollen was re-

moved from different body parts by means of small pieces of sticky gel (Beattie 1972). The pollen was identified and counted under a light microscope; reference pollen from all flowering plant species was sampled during the observation period. The second approach consisted of comparing the reproductive output of naturally pollinated with manually pollinated flowers (see below).

Reproductive success after different pollination treatments

Reproductive success was determined by counting the number of filled (=viable) and empty, shrivelled (=aborted) seeds. Post-hoc germination experiments proved the validity of this diagnosis. The sum of the viable and aborted seeds was used to estimate the initial number of ovules. Seed set is the percentage of viable seeds of the initial number of ovules.

Seed set in naturally pollinated flowers was measured in all populations and plots, while experimental pollination treatments were performed in K-1 and K-2 (18-20 August), and in O-1 (8-9 September). Fruits were collected ca. 25 days after the treatment. Recovery of the caged fruits in all cases was over 90%. Tests of self-compatibility and autofertility (spontaneous self pollination by autodeposition of pollen on the stigma) were performed on plants covered by a metal cage with fine gauze to prevent insect visitation. On each plant, buds were marked with waterproof acrylic paint and assigned to two different treatment groups: manual self pollination ($n_{K-1}=49$, $n_{K-2}=45$, $n_{O-1}=118$) or spontaneous self pollination (=no manipulation; $n_{K-1}=60$, $n_{K-2}=71$, $n_{O-1}=93$). Manual selfing was performed with pollen from the flowers' own anthers. To assess natural pollination success, we selected flowers on uncaged plants. These flowers were marked individually on the same days the other treatments were conducted, and left uncaged ($n_{K-1}=51$, $n_{K-2}=61$; $n_{O-1}=40$ and $n_{O-2}=50$). The effect of manual cross pollination was estimated on a sample of uncaged plants at Oostvoorne; flowers with freshly exposed stigmas were outcrossed by hand, marked and left uncovered until harvesting ($n_{O-1}=20$). The plants could not be emasculated, since this resulted in abortion of the flower.

All manual pollinations consisted of a single application of an abundant quantity of pollen. In all cases, we used the tallest plants within a given population, in order to facilitate flower handling. In outcrossing experiments, pollen donors were selected at a distance of 0.5 up to 2.5 m from the pollen receptors. A large number of dehisced anthers was collected in a Petri dish and subsequently rubbed over the stigmas to be outcrossed (3-5 on each stigma). Therefore, we assume that the ap-

plied pollen on a stigma was from several fathers. To investigate reproductive success, 30 fruits per treatment were selected at random from the harvested fruits.

Statistical analysis

Prior to statistical analysis, the data were tested for normality using Kolmogorov-Smirnov/Lilliefors tests (Sokal & Rohlf 1995). If normality could not be achieved by transformation, non-parametrical tests were used. Variation in reproductive success as a result of the pollination treatments was tested with either ANOVA followed by Tukey's HSD multiple comparison tests of differences among means, or by Kruskal-Wallis ANOVA followed by separate Mann-Whitney U-tests (hereafter referred to as K-W and M-W, respectively). Percentage data on seed set were angularly ($\arcsin\sqrt{x}$) transformed prior to testing (Sokal & Rohlf 1995). Statistical analyses were performed using the computer package SYSTAT 5.2 (Wilkinson 1989). All reported means are accompanied by their standard error (SE). Time is given in CET (Central European Time).

Results

Floral characteristics: morphology, anthesis, and nectar standing crop

The flowers were homogamous. At the same time, the anthers presented fresh pollen and the stigma was receptive (i.e. the two lobes were open and the papillae were visible). Also, we observed no herkogamy. The stigma lobes opened at approximately the same or a somewhat inferior height as the anthers. Flower life span in O was 2.2 ± 0.10 days (range 1-4 days, $n=49$), whereas in K-2 it was 2.0 ± 0.15 days (range 0-3, $n=31$). This difference was not significant (t-test, $P>0.05$). The flowers closed in the evening and opened in the morning. Opening and closing times depended upon the daily weather conditions (Petanidou et al., unpublished data).

Table 1 gives the mean nectar standing crop of uncaged flowers of the Kennemerduinen (K-2) and Oostvoorne populations on different days. In site K-2, closed and unvisited flowers (sampled 08.00 h) had a higher nectar yield than those sampled later in the day, which implies that nectar was soon consumed by visiting insects. This is less clear in Oostvoorne, probably because this population was not sampled as early in the morning as K-2.

Table 1. Nectar standing crop of *Gentianella uliginosa* at different sites and times of the day. Nectar quantities are means of the cumulative values ($\mu\text{l}/\text{flower} \pm \text{SE}$). Flowers of 8.00 h at K-2 were all closed.

Site	Date	Time n	Sample size μl	Nectar	SE
Kennemerduinen-2	18.8.89	8.00	16	0.127	0.029
	-	17.00	28	0.011	0.006
	19.8.89	13.00	31	0.017	0.003
Oostvoorne-1	4.9.89	15.00	43	0.054	0.030
	-	16.30	34	0.013	0.007
	5.9.89	13.00	43	0.039	0.012
	-	18.00	25	0.031	0.012
	6.9.89	10.00	42	0.055	0.018

Flower visitors - Bumblebee constancy and efficiency

Both in population Kennemerduinen and in Oostvoorne, *Gentianella uliginosa* was predominantly visited by workers of one bumblebee species, viz., *Bombus pascuorum* (Scopoli). *B. lucorum* (Linnaeus), *B. terrestris* (Linnaeus), *B. lapidarius* (Linnaeus), as well as *Apis mellifera* (Linnaeus) were also present in the neighbourhood of both localities, but these insects rarely visited *Gentianella*. In the Kennemerduinen sites, these species predominantly visited *Pulicaria dysenterica*, and in Oostvoorne *Mentha aquatica*. On the other hand, *Bombus pascuorum* majored (sensu Heinrich 1981) not only on *G. uliginosa*, but also on other species, such as *Lotus corniculatus*, *Lythrum salicaria*, *Euphrasia stricta*, and *Echium vulgare*. Syrphid flies and other potential pollinators were also observed visiting *G. uliginosa* on both localities, including *Metasyrphus luniger* (Meigen) in population Kennemerduinen, and *Syrirta pipiens* (Linnaeus), *Eristalis tenax* (Linnaeus), *Sphaerophoria scripta* (Linnaeus), and the butterfly *Aglais urticae* (Linnaeus) in population Oostvoorne. All these species very rarely visited the flowers of *G. uliginosa*. In addition, we found very few (i.e., 0-10) pollen grains of *Gentianella* on the bodies of the individuals we checked. For these reasons, we concluded that these insect species were not important as pollinators of *G. uliginosa*.

The data on bumblebee visitation (Fig. 1 a-d) show that both Oostvoorne plots were visited by a higher number of bumblebees than the K-2 plot. The total number of visits paid to *G. uliginosa* flowers in the Oostvoorne plots was smaller than in the Kennemerduinen plot (Fig. 1d). In plot K-2, a single marked bumblebee was responsible for more than 70% (76 out of 106) of all recorded visits to the *Gentianella* flowers (Fig. 1c).

The *Mentha*-dominated O-2 plot (422 *Mentha* heads and 201 *Gentianella* flowers) attracted significantly more bumblebees than the *Gentianella*-dominated (99 *Mentha* and 334 *Gentianella*) plot O-1 (Fig. 1a and b, M-W U-test, $P=0.006$). On the other hand, the average proportion of these bumblebees that visited *Gentianella* in the course of a day was significantly lower in plot O-2 (0.25) as compared to O-1 (0.45; M-W U-test, $P=0.045$). However, this did not result in a significant difference in bumblebee visitation rate per flower between both plots (on average 0.22 vs. 0.39 visits/flower in plot O-1 and O-2, respectively; M-W U-test, $P>0.10$).

In all populations, bumblebees visited the flowers for nectar, but they also collected the pollen adhering to their bodies. They landed over the flower opening, grasped the flower and pushed themselves inside the corolla tube. Because of the bumblebee's weight, the flowers turned upside down so that pollen was shed on its body. In addition, the bee's body also rubbed considerable amounts of pollen from the anthers while entering the flower. Given the position of anthers and stigma, the handling of flowers by the bumblebees are likely to increase the deposition of self pollen.

The results of the analysis of pollen loads on different body parts and in the corbicula are given in Tables 2 and 3, respectively. The largest quantities of *G. uliginosa* pollen were detected on the ventral and frontal parts of the bumblebee bodies (viz., the head, fore legs, and ventral side of the thorax, Table 2). This implies that pollen transfer and deposition mainly occurred sternotribically. In population K-2, there was a marginally significant decrease in the proportion of *Gentianella* pollen in the corbicular loads after 12:00 (M-W U-test, $P=0.073$; cf. Table 3). At the same time, the relative abundance of *Lotus corniculatus* pollen tended to increase, although this was not significant (M-W U-test, $P=0.114$). In O-1, where *Mentha* was the

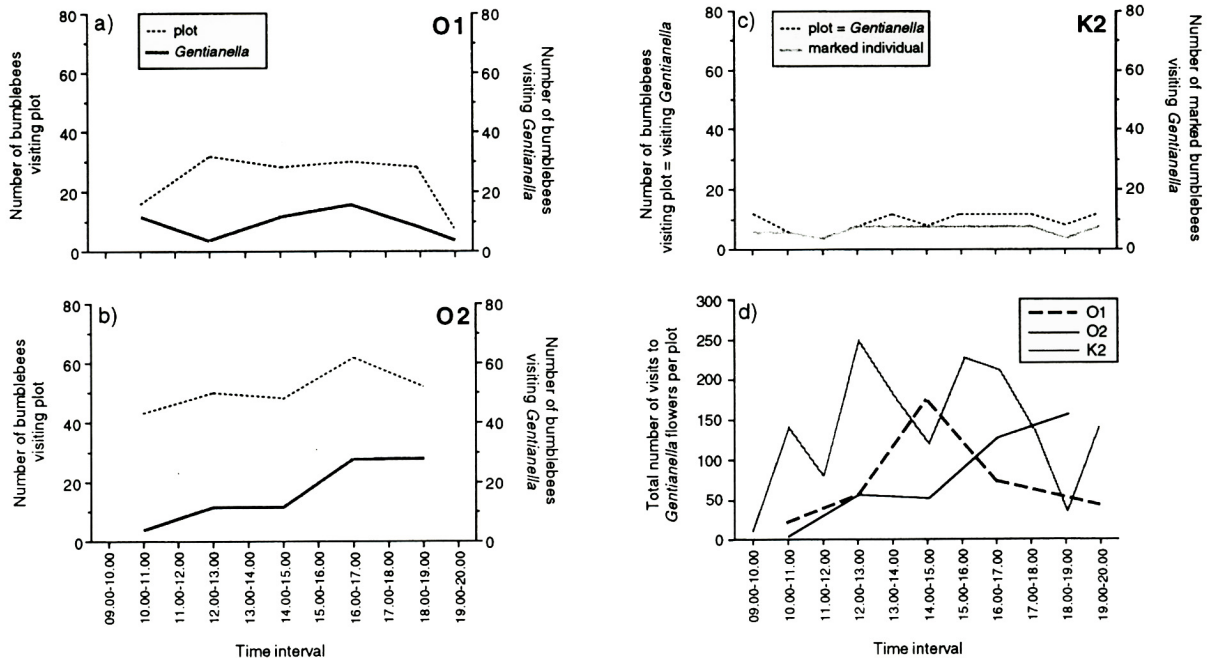


Fig. 1. Number of bumblebee visits per time interval to the plots and the *Gentianella* flowers in the plots (a-c), and the total number of visits to *Gentianella* flowers in the different plots (d) (Oostvoorne-1 and Oostvoorne-2, 4-7 August 1989; Kennemerduinen-2, 17 and 19-20 August).

main co-flowering species, there was a marginally significant increase of the amount of *Gentianella* pollen in the course of the day (M-W U-test, $P = 0.053$). During the afternoon, bumblebees carried much more *Gentianella* pollen in plot O-1 than in K-2 (M-W U-test, $P = 0.009$). There was no significant difference in pollen load composition between the O-1 and O-2 plots (i.e., with few and abundant *M. aquatica* flower heads, respectively; M-W U-test; $P = 0.935$; Table 3).

No bumblebee marked in either of the K-1 and K-2 plots in Kennemerduinen was recaptured in the other plot. This suggests that pollen flow between the two subpopulations was very limited at the most.

Reproductive success

Fruit set was more than 90% in all cases. Data on reproductive success after the various pollination treatments are shown in Fig. 2. Extended statistical results are presented in Table 4. In both Kennemerduinen sites (Fig. 2a and b), the number of ovules recovered from naturally pollinated flowers was significantly lower than the number of ovules in manually or spontaneously selfed flowers. In Oostvoorne (plot O-1), manually selfed flowers had significantly more ovules than naturally pollinated flowers (Fig. 2c).

Table 2. Composition of pollen (number of pollen grains \pm SE) carried on different body parts of *Bombus pascuorum* in Kennemerduinen-2.

Body part	Sample size n	<i>Gentianella</i> <i>uliginosa</i>	<i>Lotus</i> <i>corniculatus</i>	<i>Lythrum</i> <i>salicaria</i>	Others	Total
Fore legs	4	186 \pm 89	372 \pm 280	27 \pm 9	206 \pm 32	791 \pm 298
Head (whole)	6	240 \pm 116	218 \pm 211	46 \pm 21	53 \pm 33	557 \pm 366
Thorax (frontal)	8	131 \pm 32	659 \pm 495	93 \pm 56	138 \pm 52	1021 \pm 511
Thorax (dorsal)	8	9 \pm 2	7 \pm 4	5 \pm 4	12 \pm 5	33 \pm 11

Table 3. Composition of pollen (percentages of pollen grains \pm SE) carried in the corbiculae of *Bombus pascuorum* in Kennemerduinen-2(K-2), Oostvoorne-1 (O-1), and Oostvoorne-2 (O-2).

Plot	Sample size n	Period of day	Pollen composition				
			<i>Gentianella uliginosa</i>	<i>Lotus corniculatus</i>	<i>Lythrum salicaria</i>	<i>Mentha aquatica</i>	Others
K-2	12	morning	31.0 \pm 5.9	11.2 \pm 3.8	44.0 \pm 8.1	-	13.9 \pm 4.2
K-2	5	afternoon	12.7 \pm 2.5	41.8 \pm 16.1	23.2 \pm 10.6	-	22.3 \pm 9.4
K-2	17	overall	25.6 \pm 4.7	20.2 \pm 6.2	37.9 \pm 6.7	-	16.4 \pm 4.0
O-1	2	morning	9.1 \pm 3.5	-	-	66.9 \pm 2.5	24.0 \pm 3.5
O-1	5	afternoon	42.4 \pm 5.5	-	-	50.1 \pm 7.7	7.4 \pm 2.3
O-1	7	overall	32.9 \pm 7.3	-	-	-	12.2 \pm 4.4
O-2	5	overall	33.4 \pm 11.1	-	-	60.4 \pm 12.0	6.2 \pm 1.1

In all three plots (K-1, K-2 and O-1), manual selfing resulted in the largest number of viable seeds in comparison with spontaneous selfing and natural pollination, which had a similar viable seed production. Seed set was significantly lowest in the spontaneously selfed flowers in all three plots and varied from 79 to 85%. In K-1 and O-1, the seed set of naturally pollinated flowers was larger than that of spontaneously selfed ones, indicating that the activities of bumblebees were increasing seed set. This was not significant in plot K-2, however. Manual selfing increased seed set as compared to natural pollination only in this population. This suggests that there was some pollen limitation of seed set in K-2. In the other subpopulation, K-1, seed set after manual selfing and natural pollination was equally high.

In population O-1, naturally pollinated flowers showed a higher seed set than manually selfed flowers. Manual cross pollination, which was performed only in plot O-1, resulted in a significant lower seed set than hand selfing. However, when outcrossing was compared with the other pollination modes, the production of viable seeds was not significantly different.

In plot O-2, we studied only natural pollination. The mean number of ovules was significantly higher in this plot (62, as compared to 52 in O-1, Tukey's HSD, $P=0.012$), whereas the number of viable seeds did not differ (51 and 53 in O-1 and O-2, respectively). Seed set, however, was considerably higher in O-1 (97.1%) than in O-2 (87.8%, M-W U-test, $P\leq 0.001$).

Discussion

Reproductive system of *G. uliginosa* as compared to biennial and perennial gentians

In all populations studied, *Gentianella uliginosa* was characterized by fully self-compatible, homogamous flowers. There is no sign of herkogamy, since the anthers and stigmas attained about the same height. Moreover, in many flowers the anthers are positioned slightly higher than the stigmas. Probably as a result of the absence of temporal and spatial separation in pollen and stigma presentation, seed set after spontaneous selfing was high (Fig. 2). The possibility of auto-deposition of pollen within a flower has been reported for (the possibly conspecific) "*Gentiana Amarella* L.", by Müller (1873) and Knuth (1899).

In comparison to the perennial *Gentiana* species we have studied previously, the annual *G. uliginosa* had a much higher autofertility (80-85% as compared to c. 30%, Petanidou et al., 1995a, 1995b). Also the closely related, strictly biennial *Gentianella germanica* (Willd.) Börner exhibited a much lower seed set after spontaneous selfing (c. 30%, Luijten 1992). In contrast, Swedish (late-flowering) populations of the biennials *Gentianella campestris* (L.) Börner and *G. amarella* (L.) Börner seem to have the same high autofertility as we observed in *G. uliginosa* (c. 87-90%, Lennartsson 1997). Whatever causes the differences between the biennials is not clear, but herkogamy (occurring in *G. germanica* and *G. campestris*) may play an important role (Oostermeijer et al. 1994, Lennartsson 1997).

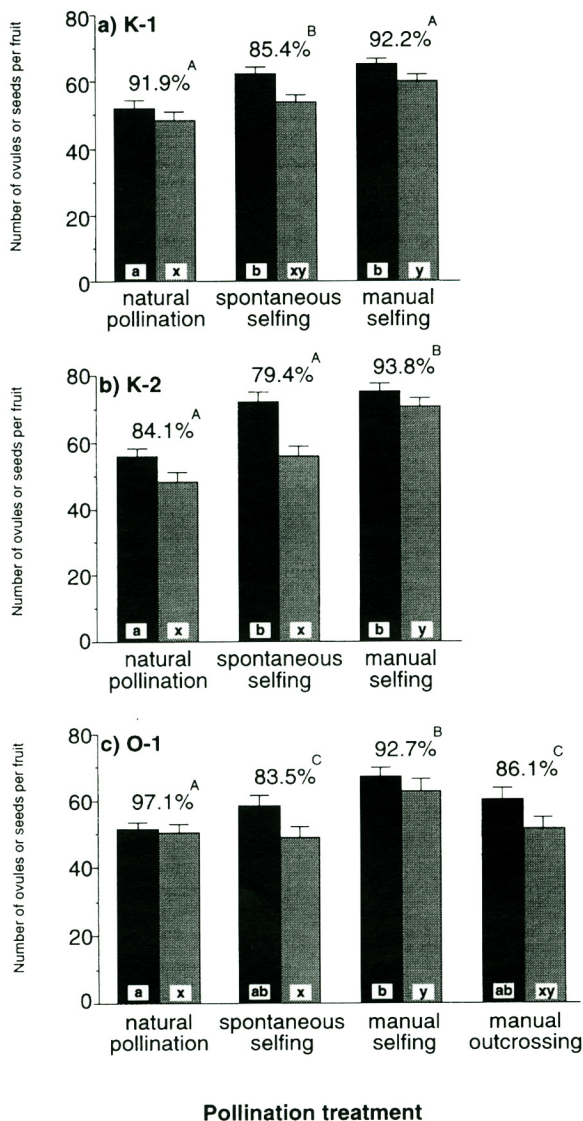


Fig. 2. Reproductive success of *Gentianella uliginosa* in the plots Kennemerduinen-1 (a), Kennemerduinen-2 (b), and Oostvoorne-1 (c). Columns represent the mean initial number of ovules (viable + aborted seeds) and the mean number of viable seeds per fruit. The resulting seed set percentages (backtransformed means) are given on top of each pair of columns. Lowercase and capital letters at the foot and above the bars summarize the results of the multiple comparison tests for initial number of ovules (a, b), number of fertile seeds (x, y, z), and seed set (A, B, C). Columns/seed set figures that have a letter in common do not differ significantly (see Table 4 for statistics). In this figure, only the statistics between treatments within sites are shown. Error bars represent ± 1 SE.

The role of pollinators

In all study sites but K-2, natural pollination led to higher percentages of seed set than spontaneous selfing. The obvious explanation for this difference is that under natural (uncaged) conditions, pollinator activity facilitates the transport of pollen within and between flowers. On the other hand, there might also have been a negative effect of the gauze-covered cage on seed set. In that case, however, seed set in the manual selfing treatment of caged flowers would have been reduced as well, but we observed the contrary. It might be argued that autodeposition of pollen was lowered in the caged, unhandled flowers because movement of the plants by the wind was inhibited. Since the study plants hardly stuck out above the surrounding vegetation, this effect is unlikely, although it cannot be fully dismissed. Still, the most plausible explanation of the difference found between open and spontaneous selfing is that pollinators indeed contribute to reproduction in the studied populations of *Gentiana uliginosa*.

Apparently, the presence of a simultaneously flowering species such as *Mentha aquatica* did not result in reduced bumblebee visitation. When *Mentha* was dominant, significantly more bumblebees were attracted, but the proportion of visits to *Gentianella* flowers was reduced since the bumblebees divided their activities between both species. Eventually, this resulted in an average visitation rate per flower that was not significantly different from the visitation rate observed in the plot where *Gentianella* was dominant. Still, seed set after natural pollination was significantly higher in plot O-1 than in the *Mentha*-dominated plot O-2 (97 vs. 87%). Together with the difference in ovule number, the final result was an equal production of viable seeds per fruit (51 vs. 53). An explanation for this peculiar observation may be an ecological difference between the plots. *Mentha aquatica* is an indicator for sites with high soil moisture conditions. This suggests that water availability for *G. uliginosa* was higher in the *Mentha*-dominated plot O-2. This might for instance have been reflected in the significantly higher number of ovules in this (62), as compared to the other plot (52). Our field observations indicate that plants in plot O-2 were also taller and had more flowers than those in plot O-1. This agrees with earlier observations on *G. uliginosa* in the Kennemerduinen, where de Haan (1990) found significantly positive relationships between vegetation height, plant size, flower number and ovule production per flower. Also in *Gentianella caucasea* (Lodd. ex Simms) J. Holub, Akhalkatsi & Wagner (1996) observed a higher ovule production in flowers on larger plants. If we assume that plants in O-2 were larger and produced more ovules as a consequence of higher availability of water, but received the same amount of bumblebee vis-

Table 4. Summary of statistical comparisons between the pollination treatments¹ in *Gentianella uliginosa*. Total number of ovules and number of viable seeds were tested using parametric ANOVA followed by Tukey's HSD multiple comparisons test; seed set percentages were compared by means of Mann-Whitney U-tests.

	Plot K-1		Plot K-2		Plot O-1		
	SS	MS	SS	MS	SS	MS	MO
Ovules							
NP	HSD=10.5 P=0.004	HSD=13.4 P<0.001	HSD=16.3 P=0.004	HSD=19.5 P<0.001	HSD=6.9 P=0.369	HSD=15.7 P=0.002	HSD=8.6 P=0.269
SS	-	HSD=2.9 P=0.636	-	HSD=3.2 P=0.706	-	HSD=8.7 P=0.177	HSD=1.6 P=0.986
MS	-	-	-	-	-	-	HSD=7.1 P=0.434
Viable seeds							
NP	HSD=10.9 P=0.219	HSD=24.0 P=0.001	HSD=15.1 P=0.145	HSD=44.7 P<0.001	HSD=1.7 P=0.982	HSD=12.4 P=0.033	HSD=1.1 P=0.997
SS	-	HSD=13.1 P=0.121	-	HSD=29.7 P=0.001	-	HSD=14.0 P=0.011	HSD=2.7 P=0.946
MS	-	-	-	-	-	-	HSD=11.3 P=0.104
Seed set							
NP	U=655 P=0.006	U=453 P=0.862	U=514 P=0.234	U=300 P=0.027	U=770 P<0.001	U=583 P=0.039	U=557 P<0.001
SS	-	U=635 P=0.006	-	U=650 P=0.001	-	U=656 P=0.002	U=279 P=0.491
MS	-	-	-	-	-	-	U=140 P<0.001

¹ NP = Natural Pollination; SS = Spontaneous Selfing; MS = Manual Selfing; MO = Manual Outcrossing

its, they might have experienced problems with developing such large numbers of ovules (many flowers with many ovules) into filled seeds. A similar effect was observed in *G. germanica*, where a population with very large plants and many ovules per flower also had lower than optimal seed set per flower, even after manual pollen addition (Luijten 1992).

The low natural seed set of K-2 suggests that pollen transfer by bumblebees was not sufficient. Nevertheless, this plot received more pollinator visits per day than plot O-1, where we observed no pollen limitation. Perhaps these high visitation rates resulted in a higher degree of outcrossing, which in plot O-2 led to reduced seed set. Unfortunately, we don't know if reduced seed set after outcrossing also occurs in K-2. This topic is discussed in the next section.

Although bumblebees readily visit the flowers of *G. uliginosa*, it is difficult to ascertain to what extent they

affect cross pollination. Judging from their handling of the homogamous flowers, they almost certainly deposit a lot of self pollen on the stigma, and probably in much larger quantities than outcross pollen. In the biennial *Gentianella germanica*, an experiment by Fischer & Matthiess (1997) showed that emasculated flowers that were exposed to natural pollinators had very low seed set, while it was high in non-emasculated controls and in manually pollinated emasculated flowers. This result suggests that pollinators are mostly transporting self pollen within flowers, although the experiment did not exclude the possibility that emasculated flowers were visited less frequently because the pollen reward was absent. Nevertheless, the results support our hypothesis that bumblebees predominantly cause self pollination in *G. uliginosa*. Additional support for high selfing rates in this species is the complete absence of allozyme variation in all Dutch populations of this species (Luijten 1992).

Reduced seed set in outcrossed flowers

In population Oostvoorne, manual outcrossing resulted in lower seed set than selfing by hand. This result may have been caused by several factors. Firstly, the way in which we have conducted our pollination experiments may have introduced reduced seed set in the outcrossed treatment (cf. Young & Young 1992). For instance, overapplication of pollen on the stigma, mechanical damage by manual pollen transfer, caging, or emasculation have all been reported to reduce seed set in some taxa (Young & Young 1992). However, except for caging, we applied exactly the same method in the manual selfing and outcrossing treatment, so the mentioned effects seem unlikely. The cages could have had a positive effect on seed set in the manual selfing treatment because they sheltered the plants and flowers from drought, rain, herbivory, nectar robbing large bumblebees etc. Reduced seed set due to various types of environmental stress was found in the related *Gentianella caucasea* (Akhalkatsi & Wagner 1996). On the other hand, since the caged spontaneously selfed flowers showed reduced seed set and the uncaged naturally pollinated flowers did not, we can probably dismiss also this effect.

Affre et al. (1995) found a deleterious effect of emasculation on seed set in *Cyclamen balearicum* Willk. However, we chose not to emasculate the flowers of *G. uliginosa*, since this required opening very young buds, which almost invariably led to a damaged flower.

The observed reduction in seed set upon outcrossing could also have been a consequence of outbreeding depression (Templeton 1986, Templeton et al. 1986, Barrett & Kohn 1991, Ellstrand & Elam 1993). There is virtually no information on what effect artificial outbreeding would have on a predominant selfer such as *G. uliginosa*. *G. uliginosa* probably has only limited seed dispersal. The related *G. germanica* – which has similar fruits, seed size and seed shape – has a dispersal distance of less than 1 m (Verkaar & Schenkeveldt 1984). This means that a rather small-scaled genetic neighbourhood structure of inbred families might easily have developed (Parkin 1979, Barrett & Kohn 1991). In that case, we very likely made crosses between such lines, and one would only expect reduced seed development if there was some incompatibility of locally adapted gene complexes (Templeton 1986, Templeton et al. 1986). However, the high variability in the position of adult individuals from year to year – a response to the strong annual fluctuations in the ground water table (de Haan 1990) – might interfere with the development of such locally adapted gene complexes. In the biennial *G. germanica*, though, Fischer (1996) observed

decreased fitness after outcrossing over longer distances than 5 m. In contrast to *G. uliginosa*, this species occurs in comparatively predictable, annually mown or grazed chalk grasslands, where the development of a structure of locally adapted genotypes is perhaps more likely.

Nevertheless, the conclusion of this section has to be that the reduced seed set after outcrossing in plot O-1 is still not very well understood, and definitely requires new experiments in more than one population of this species.

Importance for the conservation of *G. uliginosa*

As compared to the perennial (and to some extent also the biennial) gentians we studied, *G. uliginosa* appears to be less sensitive to problems associated with small population size, such as inbreeding depression and reduced reproductive success. Judging from its reproductive biology, the species is probably a predominant selfer. As a consequence of this, most of the deleterious alleles could have been purged from the genome, reducing the level of inbreeding depression (Lande & Schemske 1985, Holtsford & Ellstrand 1990, Barrett & Kohn 1991). On the other hand, selfing will have reduced the level of genetic variation in the populations to such an extent that their ecological amplitude may have been narrowed. An example of this may be the small tolerance for reduced water availability that results in the typical narrow belt of *G. uliginosa* shifting in height from one year to the next with the fluctuating groundwater table (de Haan 1990). Apparently, there is not enough genetic variation among the individuals in the population to cope with a wider range of water availabilities. For a successful conservation of this species, it is thus more important to manage its remaining sites in such a way that the population can follow the ground water table without problems. Such problems can arise when the vegetation in parts of a dune slack has (is allowed to) become too dense, for instance with *Salix repens* or *Ligustrum vulgare*. Management successes (huge increases in population size) have already been booked by clearing valley bottoms from shrubby vegetation (through mowing or mowing followed by pony grazing), in this way greatly increasing the area of suitable habitat in wet years. After such restoration management has been carried out, the often rapid increases in population size suggest that the availability of seeds is no problem for the species. From this study, we know that at least pollen limitation, although it may occur, does not have any dramatic effects on seed production per flower. Microsite differences in soil moisture and vegetation height probably have more significant effects than pollinators on seed production, through their effect on plant size and the number of flowers per plant.

Hence, the general conclusion of this paper may be that for this annual gentian species, ecological and demographical factors are most likely far more important than genetics or pollination biology. *Gentianella uliginosa* therefore contrasts strongly with the perennial *Gentiana pneumonanthe*, where small populations not only experience ecological and demographical problems, but in addition suffer from inbreeding depression and severely reduced reproductive success (Oostermeijer 1996a, 1996b).

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