Pollination ecology and constraints on seed set of the rare perennial *Gentiana cruciata* L. in The Netherlands*

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SUMMARY

Pollination ecology and components of reproductive success of *Gentiana cruciata*, a rare species in The Netherlands, was studied in two dune populations (20 and *c.* 300 flowering individuals, respectively) during the summer of 1989. *Gentiana cruciata* was fully self-compatible, but spontaneous self-pollination resulted in severe pollen limitation: natural pollination showed lower reproductive success as compared to hand-pollinations. In addition, reproductive success in the small population was significantly lower through all pollination treatments. Natural pollination was carried out mainly by the bumblebee *Bombus pascuorum* visiting the flowers for nectar, which was available at all floral stages but in greatest quantities during the morning hours. The number of visitors and visits to the flowers was generally high. However, the constancy of bumblebee visitors (measured as the proportion of heterospecific pollen found on bumblebees) decreased strongly in the afternoon, when nectar standing crop was relatively low. The number of visits and visitors to the *G. cruciata* flowers remained still very high, but the average time spent per flower decreased significantly. We argue that, in general, the low reproductive success through natural pollination is caused by pollen limitation as a consequence of competition through heterospecific pollen (mainly of the abundantly co-flowering shrub *Rubus caesius*) transferred and deposited on the stigmas by the bumblebees. The overall lower reproductive potential of the small population may be the result of a combination of poor site quality, rabbit grazing, and inbreeding.

Key-words: competition for pollination, breeding system, conservation biology, population size, heterospecific pollen, seed set.

INTRODUCTION

In general, local population size is an important factor for the chance of extinction of plant species (Barrett & Kohn 1991). Hence, the fact that many wild plant species nowadays often occur in very small and isolated populations as a result of fragmentation, destruction or deterioration of their natural habitats is alarming. Especially in small populations of outcrossing, self-compatible plant species, genetic variation is lost by genetic drift and inbreeding (Barrett & Kohn 1991; Ellstrand & Elam 1993). This 'genetic erosion', which is not counteracted by gene flow owing to the increasing isolation among populations, results in reduced fitness, which may possibly cause local extinction even if the ecological conditions are relatively suitable (Karron 1987; Ouborg et al. 1991; van Treuren et al. 1991; Ouborg 1993; Oostermeijer et al. 1992, 1994a; Raijmann et al. 1994).

Particularly in animal-pollinated and predominantly outbreeding plant species, reduced population size and fragmentation of natural habitats may have strong negative effects on the reproductive output (Jennersten 1988; Kwak 1988; Kwak et al. 1991; Petanidou et al. 1991; Huenneke 1991; Oostermeijer et al. 1992; Jennersten & Nilsson 1993). 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). On the other hand, these small populations may be especially prone to reduced seed set because of the presence of such neighbours, as a result of competition either for pollinators (Mosquin 1971; Kwak & Jennersten 1991) or through heterospecific pollen transfer (Waser 1978, 1983; Thomson et al. 1981; Rathcke 1983; Campbell & Motten 1985).

As part of a comprehensive study of all rare *Gentiana* and *Gentianella* species in The Netherlands, this paper deals with the pollination ecology, reproductive biology and breeding system of the herbaceous perennial *Gentiana cruciata* L. which is a very rare species in the Dutch coastal dune area. In two of the four presently existing populations (one very small and one larger, but still relatively small) the reproductive potential, dependence upon the native pollinating fauna and the interactions with the co-flowering species are studied. With respect to pollination ecology, *G. cruciata* is very similar to its congener *G. pneumonanthe* L., a species of wet heathlands and hay meadows. In previous studies it has been demonstrated that the latter species strongly depends on pollinator visits for a high seed production (Petanidou et al. 1995). Also, the lack of seed germination and seedling survival have proved to be a major problem in small populations of *G. pneumonanthe* (Oostermeijer et al. 1992, 1994b), and reproductive success and offspring fitness are related to population size and genetic variation (Oostermeijer et al. 1992, 1994a).

MATERIALS AND METHODS

Biology of the species

Gentiana cruciata (Gentianaceae), the cross gentian, is a herbaceous perennial plant with several stems of up to 40–50 cm high. According to Meusel et al. (1978), the species has a Euro-Westsiberian, submeridional/montane distribution with some outposts found in The Netherlands. In the latter country, at the margin of its distribution range, *G. cruciata* has always been a very rare species that is only found in the calcareous part of the coastal dune area. The distribution pattern has been rather stable in the sense that
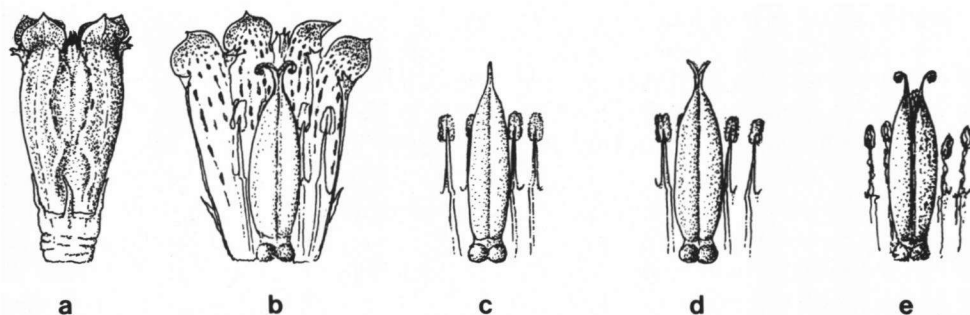
Fig. 1. Flower of *Gentiana cruciata* in different stages: (a) outer side, (b) female stage in longitudinal section. Details: (c) male, (d) early female, and (e) mature fruit with withered stamens. Drawing by J.G.B. Oostermeijer.

the loss of some localities has been compensated by the gain of some new ones since 1950. At present only four populations occur in The Netherlands, ranging in size from 10 to several thousands of flowering individuals. The actual distribution may have been affected by the importation of pheasant fodder in historical times (Mennema *et al.* 1980)

In Figure 1, the morphology of the flower and the different stages of the anthesis are shown. The flowers of *G. cruciata* are of the funnel type, borne mainly in one terminal, and secondarily in two to three stem whorls. The floral tube is *c.* 18 mm deep. An average stem of a mature, generative plant may bear up to 50 flowers in dense clusters. The central flowers of these clusters are in most cases atypically 5-lobed (also bearing 5-stamens) and are larger than the peripheral, 4-lobed flowers. The corolla lobes are blue to light mauve on the inner side and slightly reflect UV light, whereas the small corollary appendages, located in the sinuses of the lobes are strongly UV-reflecting (Petanidou *et al.* unpublished data). At their base, the corolla lobes become white, and the whole construction forms a clear white ring around the entrance of the floral tube. Inside the corolla, this nectar guide ring continues in a pattern of parallel white and lilac stripes that guide pollinators to the nectar that is produced at the base of the ovary.

Study sites

All field work was performed during the summer of 1989. Two study sites, located in the dune area at the western coast of Holland were selected.

The first population (hereafter mentioned as 'Castricum') is situated near Castricum, province of North Holland, *c.* 15 km SW of Alkmaar. The population, situated within the nature reserve 'Noordhollands Duinreservaat', is assumed to be of rather recent spontaneous origin, dating from 1945–1950 (Mennema *et al.* 1980). At the time of this study, the population was divided into two subpopulations with a distance of about 100 m, and consisted in total of 20 flowering and a few juvenile and mature vegetative individuals. In the beginning of the growing season, 10 plants were selected and fenced, to prevent the plants from being eaten by rabbits. Fence heights did not exceed flower heights so that flowers could be seen and visited by insects. The fences were kept throughout the study period until the seeds were harvested. Important co-flowering species at the site were *Rubus caesius* L. (Rosaceae), *Cirsium vulgare* (Savi) Ten., and *Senecio jacobaea* L. (Compositae), and *Echium vulgare* L. (Boraginaceae).

In the Castricum population, we performed observations on flowering phenology and a series of pollination treatments to determine reproductive potential and success.

The second population (hereafter called 'Oostvoorne') is situated in a nature reserve (Voorne's Duin) managed by the Dutch Society for Preservation of Nature near Oostvoorne, province of South Holland, c. 20 km SW of Rotterdam. The origin of this population is not yet certain. It may have been founded by seeds imported from a botanical garden (J. van Baarsen, personal communication). During this study the population was estimated to consist of c. 300 flowering and several hundreds of seedlings, juveniles and mature vegetative individuals occupying a total area of approximately 1 ha. The core of the population (c. 80% of all individuals) was restricted to an area of c. 30×8 m^2. The main co-flowering species at this locality were *Rubus caesius, Senecio jacobaea, Cirsium vulgare, Oenothera biennis* L. (Onagraceae), and *Thymus pulegioides* L. (Labiatae).

In addition to the aspects studied in Castricum, here also some particular elements of plant–pollinator relationships were studied: flower and flowering characteristics, nectar production, pollinator behaviour, and pollination effectiveness, the latter in relation to the co-flowering species. All experimental fieldwork was performed during the peak of the flowering period, from 2 to 10 August 1989, i.e. a total of 11 days.

Determination of flowering characteristics

Phenology of *G. cruciata* was recorded in the Castricum population on all 10 fenced plants by counting the number of open flowers every 4–10 days during the flowering period (mid-July–mid-August 1989). Since we noticed that the flowers close over night and they open and may close again in the course of the day, we also studied the daily opening pattern of flowering in the Oostvoorne population. We selected two plots (4×1, and 0.4×0.4 m^2) in the area with the highest plant density. The number of open flowers in both plots was counted every 30 min throughout 6 days (3–4 and 6–9 August). At the same time, the air temperature and relative humidity were recorded. The flower life span was measured on 61 flowers borne on different plants, occupying different positions within the inflorescence. The flowers were colour-coded with acrylic paint at the bud stage during the peak of the flowering period, and individually monitored three times per day until they withered. To determine the initial number of ovules, 12 fresh, unpollinated flowers were sampled at random from the field and the number of ovules per flower was directly counted under a dissecting microscope.

Nectar measurements

In the Oostvoorne population, the amount of nectar in the flowers, expressed as volume, was measured by inserting a 1 µl Drummond microcapillary into the corolla tube in order to collect the nectar produced at the ovary base. Two different sets of nectar measurements were made. The first served to establish nectar production rate at different stages of the flowers. Nineteen flowers at bud stage were marked and covered by a metal cage with fine gauze to prevent insects from removing the nectar. Throughout their entire anthesis, nectar was sampled twice a day in the male phase, and once a day during the female phase, when nectar proved to be scarce. The second measurement concerned the nectar standing crop in open-pollinated flowers. To determine this, the nectar was harvested from a number of open flowers of different stages at three different times of the day. Flowers were selected at random from plants close to the caged ones.

Observations on the behaviour of pollinators

On 4, 6, 8 and 9 August, the behaviour of flower-visiting insects was monitored in the 4×1 m^2 plot of the Oostvoorne population. During the first 2 days, when flowers were open the plot was systematically monitored for each parameter, for 15 min every hour (11 times per day). Behavioural parameters recorded were (i) the number of visitors entering the plot and consequently visiting gentians, (ii) the number, and (iii) the sequence of visits to the different flower stages, as well as (iv) the bumblebee average handling time per visit.

The pollination effectiveness of the bumblebees was assessed by means of the following procedures.

1. The (con- and heterospecific) pollen loads borne on the different bumblebee body parts and in the corbiculae were sampled. To a very limited extent this method was also applied for assessing the pollination efficiency of other, less frequent visitors, such as sweat bees (Halactidae) and hoverflies (Syrphidae). The loads were sampled from insects captured between two consecutive visits to *G. cruciata* or in the vicinity of the study plot during the study period. The visitors were lightly anaesthetized by means of ethyl-acetate, and the pollen from the different body parts, as well as from the corbiculae, was removed by using a small sticky piece of gel (Beattie 1972). The pollen was identified and counted under a light microscope using reference pollen material from co-flowering species in the field.

2. The amount of pollen grains deposited on *G. cruciata* stigmas after a single bumblebee visit was counted. Four well-exposed virginal flowers that had been caged until their fourth day of female stage were uncovered and allowed to be visited once by a bumblebee after this had visited several other conspecific flowers (14.00 h, 8 August). After this single visit, the stigmas were collected and stored on slides until further examination in the laboratory (Beattie 1972; cf. above).

3. The reproductive success through natural pollination was compared with that through hand and spontaneous pollination, so the contribution of flower visitors to pollination could be determined (see under the description of the pollination experiments).

Pollination treatments and determination of reproductive success

Three different pollination treatments, hand selfing, hand outcrossing and spontaneous selfing, were performed in both study populations, from 17 to 26 July in the population Castricum, and from 4 to 9 August in the population Oostvoorne. The pollination treatments were performed as follows.

Hand selfing. Plants ($n_{Castricum}=10$, $n_{Oostvoorne}=30$) were covered by a metal cage with fine gauze to prevent insect access. In Castricum only parts of the plants were covered, the rest left open to be freely visited by insects. The flowers that were to be treated ($n_C=93$, $n_O=92$) were marked at the bud stage with a specific colour of acrylic paint. A few ripe stamens were removed from the flower with fine forceps and rubbed gently over their own receptive stigma, applying, in this way, an abundant quantity of pollen on the flower stigmas.

Spontaneous selfing. On the same caged plants as in the previous treatment, flowers ($n_C=112$, $n_O=178$) received a different colour code at the bud stage and were

consecutively left untouched to make sure all pollen transfer occurred spontaneously within the flower.

Hand outcrossing. Plants were left open to visitation by pollinators and flowers ($n_C=36$, $n_O=87$; on 10 and 45 plants, respectively) were marked at the bud stage with acrylic paint at the same time as the preceding two treatments. Cross-pollination was performed manually, by rubbing a few ripe anthers of another plant from the same population over the receptive stigma.

For a comparison of the pollination treatments with the reproductive success through natural pollination, fruits of open-pollinated flowers ($n_C=71$, $n_O=62$; on the same 10 and 45 plants as in hand outcrossing, respectively) were also harvested. The flowers had received a specific colour code at the bud stage at the same time as the experimental treatments.

The fruits were harvested approximately 1 month after pollination. Recovery of the fruits was very high, and dropped only in the case of freely exposed flowers as a result of predation, though it was never lower than 90%. As a standard procedure, 30 fruits per treatment, selected at random from the total number of fruits that had been collected, were used. Reproductive success was determined by counting the number of mature seeds present in each fruit. Two types of seeds were found: minute brownish granules (=unfertilized ovules) to empty, shrivelled (=aborted) seeds; and filled seeds. Germination experiments showed that only the seeds of the latter group were viable (>90% germinability). They will be referred to as viable seeds contrasting to aborted seeds (=unfertilized ovules and aborted seeds). The sum of the viable and aborted seeds will be considered here as representative for the initial (i.e. total) number of ovules.

In the exposed coastal dunes the wind may play an important role as pollen vector, so we tested both populations for anemophily during both day and night. On the morning of July 26, in population Castricum, 21 glass slides were placed around and between the potential pollen-donating plants at different heights (0–25 cm) and angles (0–90°) before the flowers opened, and removed 24 hours later. In population Oostvoorne, wind-pollination was tested during the daytime by using 28 glass slides (4 August). Overnight wind-pollination was also tested here as a control, since the gentian flowers are closed during the night.

Statistical analysis

Prior to statistical analysis, the data were tested for normality (Kolmogorov-Smirnov/ Lilliefors tests). To meet the assumptions for parametrical tests, the data were either log-transformed (number of viable seeds) or angular-transformed (seed set and percentages of pollen load). In cases where transformation did not improve normality, 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. Spearman's rank correlation coefficients were calculated between air temperature, relative humidity and the number of open flowers in the plot. Partial correlation coefficients (Sokal & Rohlf 1981) were calculated to determine the relative importance of the two former parameters on the opening of the flowers.
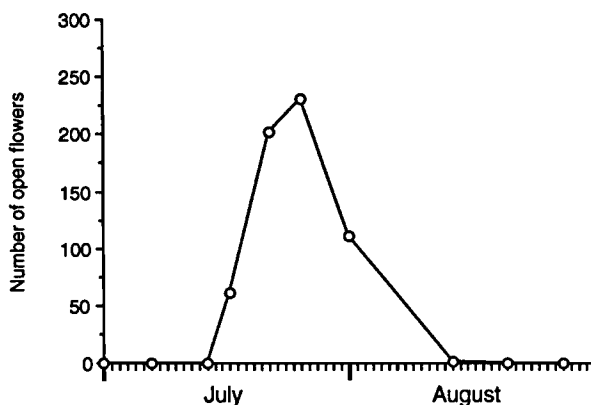
Fig. 2. Flowering phenology of *Gentiana cruciata* in the Castricum population, summer 1989. Given is the total number of open flowers in the population.

RESULTS

Flowering phenology

The flowering periods of both study populations were relatively short, which is also related to their small population sizes. The Castricum population was in flower from 15 July until 12 August 1989. Its flowering phenology is given in Fig. 2. The Oostvoorne population started flowering at 10 July (J. van Baarsen, personal communication) and lasted until 20 August.

Description of the anthesis

Anthesis in the flower clusters starts from the central flowers which thus proceed to the peripheral ones. The flower is strongly protandrous. During the male phase, the four (or five) anthers are bent against the gynoecium; after removal of the pollen they gradually bend towards the walls of the corolla tube and the female phase starts. The average total flower life span was 3.21 ± 0.11 ($n=61$, range $1.0–5.4$) days. The male stage, i.e. pollen exposure, lasted an average of 1.37 ± 0.06 ($0.3–2.7$) days, plus 0.40 ± 0.06 days during which the style was developing (hereafter called 'transition'). The 'true' female phase of anthesis lasted an average of 1.44 ± 0.09 (range $0.7–3.3$) days, when the unfolding two-lobed stigma exposed its receptive papillae. The duration of anthesis of the large flowers in the centre of the clusters was longer than that of the regular flowers (3.8 days, vs. 3.1; ANOVA, $F_{[1,48]}=4.93$, $P=0.031$).

In the evening the flowers closed and remained closed overnight. They started opening in the morning, the optimum observed (in number of open flowers), as a general rule, in the early afternoon, depending largely on inclement weather conditions (cf. Fig. 5). Their opening is negatively correlated with relative humidity (Spearman's $\rho= -0.810$, $P \leq 0.01$) and positively with air temperature ($\rho=0.553$, $P \leq 0.01$). At the same time temperature and relative humidity are (negatively) correlated ($\rho= -0.597$, $P \leq 0.01$). From the partial correlation coefficients, it follows that relative humidity is the most important parameter determining the number of open flowers (partial $\rho= -0.718$, $P \leq 0.01$), since the partial correlation of the number of open flowers with temperature is no longer significant (partial $\rho=0.148$, NS).
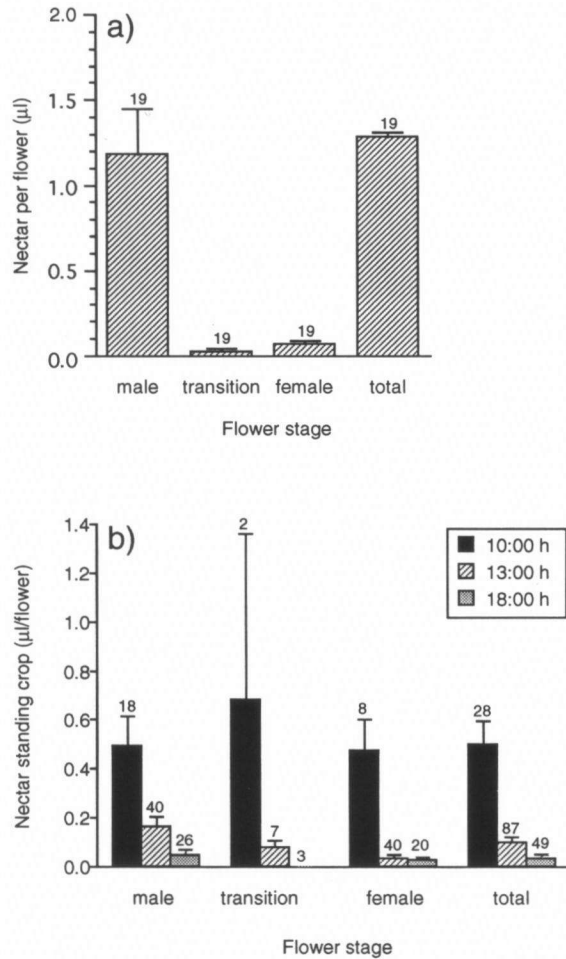
Fig. 3. Nectar production in the Oostvoorne population. (a) Nectar production pattern throughout the different flower stages and the total life span of caged flowers of *Gentiana cruciata* (mean volume ± SE). Nectarless flowers, if any, are included in the calculation. (b) Nectar standing crop of the different flower stages and of the total life span, sampled in the morning and in the afternoon (9 and 6 August 1989, respectively). Standard error and sample numbers are given on top of each bar.

Nectar production

All 19 caged flowers examined produced nectar. Their average total yield was 1·28 ± 0·14 μl. Figure 3a gives the average amount of nectar produced in the different flower stages. For the calculations, both nectar-producing and nectarless flowers of the same phase were taken into consideration. Apparently, nectar was produced mainly during the male stage (17 producers out of the 19 total). Only three flowers, of which one did not show a true male phase, produced nectar in their female stage, and only two produced nectar during the transition stage.

Mean nectar standing crop was very high in the morning hours, and declined in the course of the day for all flower stages (6 and 9 August; Fig. 3b).

Table 1. Number (mean, SE, range, number of samples examined, and composition) of pollen grains carried on different body parts of insect visitors captured in flight, after having visited a *G. cruciata* flower in Oostvoorne population. Data were taken on 3–9 August 1989, between 09.00 and 18.30 h. C, *Cirsium vulgare*; S, *Senecio jaccobaea*; O, *Oenothera*; L, Labiatae; etc, others

| | Total number of pollen grains | | | | Species | | |
	Mean	SE	Range		*Gentiana* (% of total)	*Rubus* (% of total)	Main other
Bombus pascuorum							
Head	3168	2316	186	7728	3	92·3	5·5	C
Front thorax	2559	660	453	6272	9	83·8	14·8	C, L
Hind thorax	158	36	47	342	9	71·5	22	S, L, C
Front abdomen	611	354	357	864	2	83·5	10·9	C
Hind abdomen	59				1	83·1	8·5	—
Hover flies								
All body parts	133	113·6	2	205	3	46·6	3·6	C, L, S, etc
Butterflies								
Head, legs	13	4	9	17	2	57·2	14·4	O, etc

Flower visitors

In the Oostvoorne population, *G. cruciata* was predominantly visited by workers of one bumblebee species, viz. *Bombus pascuorum* (Scopoli), which in this region occurs in polymorphic populations, composed of individuals with the phenotypes of the sub-species *floralis* (Gmelin) and *romanioides* Krüger (Apidae). Also *Bombus lucorum* (Linnaeus) and *B. terrestris* (Linnaeus) were recorded, but only at a very low frequency. *Bombus pascuorum* visited the flowers for nectar that was accumulated around the ovary base. They collected the nectar via the four openings left between the connate stamen bases. In the course of this event the insects touched the dehisced anthers sternotribically, and were dusted with pollen. During the female stage, however, when the anthers have bent towards the wall of the floral tube, the bumblebee contacted the by then unfolded stigma and its receptive papillae sternotribically (Fig. 1). Nototribical contact was very rarely observed. Our data on the distribution of pollen on bumblebees provide additional evidence for this, since *G. cruciata* pollen appeared to be most abundant on the front parts of the body, suggesting that the most effective mode of pollen transfer is sternotribical (Table 1).

Bumblebee visitors and visits to *G. cruciata* were numerous during our field observations (Fig. 4a). During their visits to flowers, bumblebees were observed seeking for and harvesting only nectar; they were never observed collecting pollen actively. Nevertheless, a large quantity of *G. cruciata* pollen adhered to their bodies (cf. Table 1) and was consecutively groomed and packed in the corbiculae during flight and occasionally during their visit.

Amongst the other, generally occasional visitors, the very common hover flies *Scaeva pyrastri* (Linnaeus) and *Sphaerophoria scripta* (Linnaeus) (Syrphidae) visited the flowers for pollen as indicated by the consumption of pollen from the anthers; the butter-flies *Gonepteryx rhamni* (Linnaeus) (Pieridae), *Coenonympha pamphilus* (Hufnagel) (Satyridae) and *Hesperia comma* (Linnaeus) (Hesperiidae) were less regular visitors, while *Halictus confusus perkinsii* (Blüthgen) (Halictidae) was observed only once on the
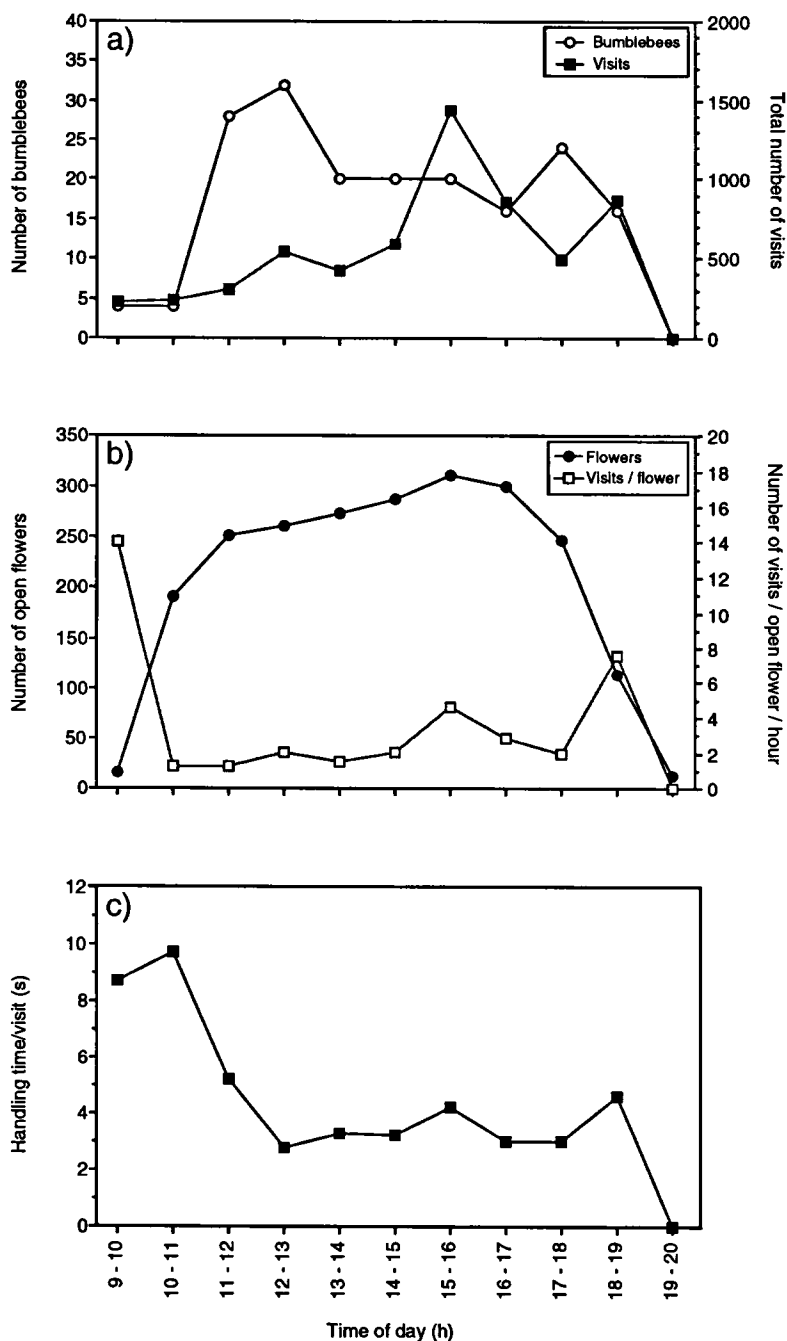
Fig. 4. Bumblebee visitation rate per hour to the Oostvoorne plot in the course of the days 4 and 6 August 1989. (a) The number of bumblebee visitors (individuals of *Bombus pascuorum*) and their total number of visits; (b) the number of open flowers of *G. cruciata*, and the number of visits per flower; (c) the mean handling time per visit (on the basis of 725 and 297 *B. pascuorum* visits recorded during the two respective monitoring days).
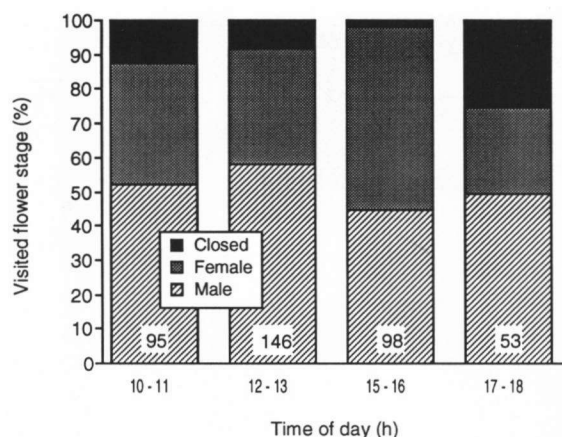
Fig. 5. Distribution of the anthesis stages of the flowers visited by bumblebees in the Oostvoorne plot throughout the day (6 August 1989). Total numbers of flowers visited are given at the foot of each bar.

flowers. The importance of all these insect species for the pollination of *G. cruciata* is probably very little, not only because of their limited visitation frequency, but also because of their extremely low body pollen loads (Table 1). In addition, *Halictus confusus* cannot be characterized as an important pollinator, not only because of its few visits to *G. cruciata*, but also because of its small size. The importance of *Bombus terrestris* for the studied populations is also questionable, since it seldom visits *Gentiana*.

The bumblebee visitation rate of the small population in Castricum was not comparable to that of Oostvoorne. During the 20 hours of monitoring, only one bumblebee was observed visiting a few flowers of the study species in the Castricum population. There were generally much less bumblebees foraging in the neighbourhood as compared to the Oostvoorne site, although the co-flowering species—especially *Rubus caesius*—were more regularly visited by *Bombus terrestris* and *B. lapidarius* (Linnaeus).

Flower constancy and bumblebee behaviour
Apparently, the pollinators in the population Oostvoorne preferred *G. cruciata* over other entomophilous species. This appears firstly from the high number of bumblebees visiting the population and the total number of visits to each open flower per day (Fig. 4a–b) and, secondly, from the behaviour of bumblebees in the morning and evening hours, when they kept visiting closed flowers by prying apart the corolla lobes and forcing themselves a way in.

The number of open flowers was significantly positively correlated with the number of bumblebee visitors in the study plot ($r=0\cdot741$, $P=0\cdot009$; cf. Fig. 4b,a) and marginally significant with the total number of bumblebee visits to the flowers ($r=0\cdot567$, $P=0\cdot070$; cf. Fig. 4b,a). The latter regression is higher when the records for the 18–19 h time interval are omitted from the analysis ($r=0\cdot693$, $P=0\cdot026$). This is caused by the fact that a very high percentage of the visits was paid to closed flowers during that time of the day (see above and in Fig. 5). It should be noted that male and female flowers occurred in equal frequencies at any time of the day; out of the 61 flowers studied, 39 started their anthesis after 12.00 h and again, 39 reached the female phase in the afternoon.
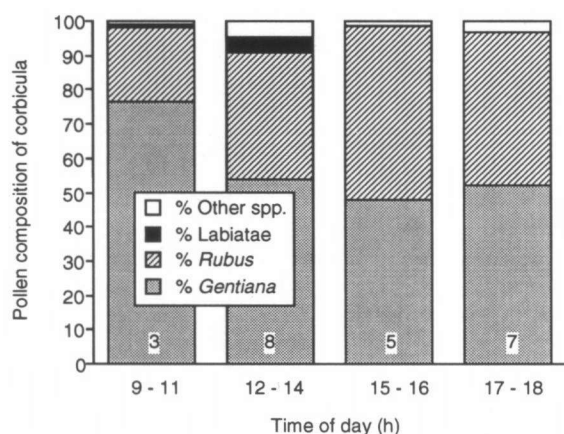
Fig. 6. Pollen composition of the corbicula loads of *Bombus pascuorum* visiting *G. cruciata* throughout the day in the Oostvoorne site. Given are the percentages of the pollen source species. The percentages for *Rubus caesius* pollen range between 4·6–56·7, 4·2–92·7, 22·5–90.3, and 6·9–90.8% for the four time intervals studied. The number of sampled individuals per time interval is given at the foot of each bar. The samples were taken on 2–4 and 6–9 August 1989.

The variation during the day in the mean time spent by the bumblebee visitors on individual *Gentiana* flowers is given in Fig. 4c. The average handling time was very long in the morning (8.00–9.30 h) and became shorter around noon (11.30–13.00 h: *t*-test, $t_{[53,186]} = 15 \cdot 529$, $P \leq 0 \cdot 001$) and in the afternoon (15.00–18.00 h; $t_{[53,41]} = 16 \cdot 280$, $P \leq 0 \cdot 001$). The mean handling times of the 11.30–13.00 h and the 15.00–18.00 h time intervals are not significantly different ($P \geq 0 \cdot 05$).

Monitoring of the visitation sequence in the Oostvoorne study plot showed that bumblebees were highly constant to *G. cruciata* flowers. However, it should be noted that the study plot, as well as the core part of the population, was fairly dominated by the species, so the demonstrated constancy may be a biased result of necessity rather than of true fidelity. In fact, from the raw data on the pollen load of individual bumblebees (as concisely presented in Table 1 and Fig. 6) the percentage of *Gentiana* pollen was incidentally as low as 4·1%. Clearly, the pollinators were not highly constant to *G. cruciata*, and even after several visits to *Gentiana* flowers they still carried considerable amounts of heterospecific pollen on their bodies (see paragraph on efficiency below). Constancy was pronounced only during the morning hours, when the flowers have just opened and the nectar standing crop was relatively high; consequently, constancy to *G. cruciata* was lower in the afternoon hours, when the nectar reward was low (Figs. 3b & 6).

Pollinator efficiency

Table 1 gives a rough estimate of a bumblebee's capacity to transfer *G. cruciata* pollen. Pollen transfer occurred mainly via the head and the front thorax.

The capacity of a bumblebee for pollen deposition was also very high: single worker visits to virginal stigmas of non-emasculated flowers resulted in an average deposition of 2597 ± 486 *G. cruciata* pollen grains (*n*=4). However, even under these circumstances, and despite the fact that the bumblebees had paid several visits to other conspecific flowers in the population before the experimental visit, considerable amounts of
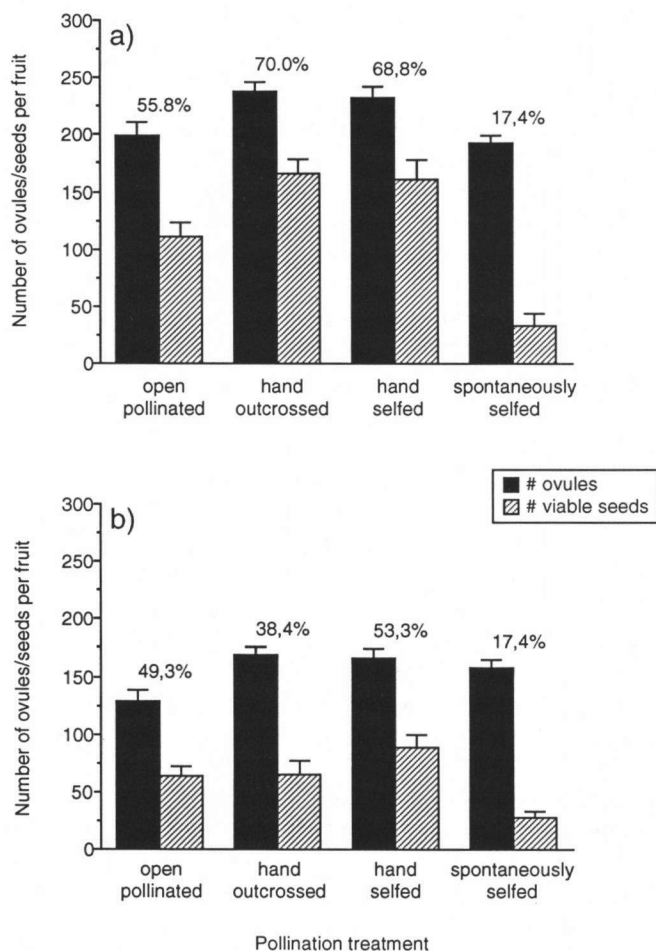
Fig. 7. Reproductive success of *Gentiana cruciata* in (a) the Oostvoorne and (b) the Castricum population for all pollination treatments, estimated over 30 fruits per treatment. Columns represent the mean initial number of ovules (viable + aborted seeds) and the mean number of viable seeds per fruit. The seed set (% of viable seeds over the initial number of ovules, backtransformed means) is given on top of each pair of columns. Error bars represent 1 unit of standard error.

heterospecific pollen were also deposited on the stigma. This heterospecific pollen came mostly from *Rubus caesius*, reaching up to 13·5% of the total deposition.

Reproductive success

All tests for anemophily showed that wind played no role in the transfer of *G. cruciata* pollen. The number of pollen grains found on the test slides was $1·0 \pm 1·96$ during the day ($n=28$) and $2·1 \pm 2·93$ at night ($n=9$) for the Oostvoorne and $0·76 \pm 0·28$ during a day ($n=21$) for the Castricum population.

The data on reproductive success per population are given in Fig. 7a and b. Fruit set was 100% in all cases examined. There was significant variation in the number of viable seeds per fruit as a result of the four different pollination treatments in both study populations (Kruskal-Wallis test: $H=20·796$, df=3, $P \leq 0·001$, and $H=45·653$, df=3, $P \leq 0·001$ for the Castricum and Oostvoorne populations, respectively).

Population Castricum

In the Castricum population, spontaneous self-pollination yielded significantly fewer viable seeds than the other treatments (Mann-Whitney U-tests; for all cases $815 < U < 1105$, $P \leq 0.05$), whereas there was no difference in the number of viable seeds among the open, hand cross, and hand self-pollination treatments (MW U-tests; $P \geq 0.072$). The same results were observed for seed set.

There was significant variation among the four pollination treatments regarding the initial number of ovules (aborted + viable seeds) per fruit (ANOVA; $F_{[3,141]} = 4.878$, $P = 0.003$). The main difference appeared to be the significantly lower mean number of initial number of ovules in open-pollinated as compared to hand-pollinated flowers (Tukey's HSD multiple comparisons, $P \leq 0.01$) and to a lesser degree also as compared to spontaneously selfed flowers ($P = 0.07$).

Population Oostvoorne

In the Oostvoorne population, spontaneous selfing resulted in significantly fewer viable seeds per fruit as well (MW U-tests; $683 \leq U \leq 780$, $P \leq 0.0001$). However, in this population also the mean number of viable seeds of hand-pollinated flowers was significantly higher than that of open-pollinated flowers (MW $U_{[1,52]} = 180.5$, $P = 0.001$, and MW $U_{[1,54]} = 252.5$, $P = 0.024$, for comparisons with hand cross and hand self-pollinations, respectively). No difference was found in the number of viable seeds between hand cross and hand self-pollination (MW $U_{[1,56]} = 407.5$, $P \leq 0.05$). Similarly, seed set of cross- and hand-selfed flowers was highest (MW U-tests; $P \leq 0.01$), intermediate in open-pollinated flowers and lowest in spontaneously selfed flowers ($P \leq 0.01$).

Also, in this population the initial number of ovules (aborted + viable seeds) per fruit showed significant variation among the four pollination treatments (ANOVA; $F_{[3,110]} = 6.322$, $P = 0.001$). Open-pollinated fruits contained significantly fewer ovules than hand cross-pollinated ones, whereas the difference with hand self-pollination was marginally significant (Tukey HSD multiple comparisons tests; $P = 0.025$, and $P = 0.060$, for cross and self-pollination, respectively).

Virginal flowers, collected in the Oostvoorne population prior to any pollination, contained an average number of ovules of 285.5 ± 10.0 ($n = 12$). This was significantly higher than the initial number of ovules recorded in ripe fruits from all pollination treatments in this population, although there was less difference between fresh flowers and fruits produced through hand-pollination (difference in means c. 50 ovules, Tukey's HSD, $P \leq 0.001$) than there was with fruits resulting through open and spontaneous pollination (difference c. 90 ovules, $P \leq 0.035$ and $P \leq 0.012$ for hand cross and hand self-pollinations, respectively).

Comparison of reproductive success between the study populations

There are differences between the populations for all investigated reproductive parameters, viz. the number of viable seeds, the initial number of ovules, and seed set (Fig. 7). The number of viable seeds is much higher in the Oostvoorne population after open-pollination and hand cross and self-pollination treatments (ANOVA, in all cases $P \leq 0.004$). This difference was not found for spontaneous self-pollination ($P = 0.569$). In all four pollination treatments, the initial number of ovules in the Oostvoorne population was higher than in the Castricum population (ANOVA, for all cases

$P \leq 0.002$). Seed set after hand-pollination treatments was higher in the Oostvoorne population (MW $U_{[1,64]} = 294$, $P = 0.002$, and MW $U_{[1,68]} = 398.5$, $P = 0.017$, for cross and self-pollinations, respectively). The seed set resulting from spontaneous selfing and open-pollination was the same in both populations, however (MW $U_{[1,63]} = 531$, $P = 0.934$ and MW $U_{[1,56]} = 343$, $P = 0.254$, respectively).

DISCUSSION

Aspects of the plant–pollinator partnership

The flowers of *G. cruciata* in the study population were intensively visited throughout the day mainly by bumblebees that paid *c.* 16 visits to each open flower during the daily opening peak between 10.00 and 18.00 h (cf. Fig. 4b). An indication of their preference for *Gentiana* is the fact that these insects kept on visiting even closed flowers. This behaviour has also been observed on other typical bumblebee-pollinated species, amongst them gentians, such as the marsh gentian, *Gentiana pneumonanthe* (Petanidou *et al.* unpublished data) and on *Gentiana andrewsii* Griseb., a closed gentian in North America which has flowers that do not open at all (Heinrich 1979a; Costelloe 1988). The number of bumblebee visitors as well as their visitation rate was proportional (the latter, roughly proportional) to the number of open flowers available (cf. Fig. 4), suggesting competition between bumblebees for *Gentiana* nectar. Bumblebees were thus the principal pollinators. Hoverflies were also observed visiting the flowers, but only for pollen; due to their poor ability to transfer pollen and the very limited number of visits, syrphids may be excluded as important pollinators of *Gentiana* in the populations studied.

Bumblebees visited the flowers for both pollen and nectar. The latter seemingly constituted a very important clue in this mutual relationship because its availability played a role in influencing the bumblebee mobility, and thus the pollen dispersal. In the morning hours, for instance, when flowers (open or closed) abundantly offered nectar, the handling time was relatively long. In the course of the day, when nectar became more scarce, the handling time decreased, but the total number of visits to the plot increased.

Our data showed that besides nectar, the pollen of *G. cruciata* was also removed and collected in large quantities. However, the bumblebee colonies in the study area did not gather pollen from *Gentiana* only (cf. Fig. 6). Apparently, during the afternoon and evening hours, when the nectar standing crop of *Gentiana* decreased, the foraging of bumblebees on other species, especially on the cornucopiously nectar-producing *Rubus caesius* (Free 1970; Proctor & Yeo 1973) which flowered abundantly in the direct surroundings, became more intensive, reducing the proportion of *Gentiana* pollen in the corbiculae.

Reproductive success and pollination limitation

Evidently, pollen transfer by bumblebees was indispensable for the reproduction of *G. cruciata* in nature, since there was no wind-pollination, and the seed set of spontaneous self-pollination was limited (cf. Fig. 7).

Since in both study populations hand cross and hand self-pollinations showed the same reproductive success in terms of seed set, it can be concluded that there is no inbreeding depression occurring at this stage. In some species, inbreeding has been

shown to increase the proportion of seed abortion (Waser & Price 1991). In most plants, however, inbreeding depression is significant only in later stages of the life history (Barrett & Kohn 1991), which agrees with the data on *G. cruciata*.

The Oostvoorne population suffered from a reduced reproductive output under natural conditions, as open-pollination showed a reduced seed set and number of viable seeds per fruit as compared to hand-pollinations (Fig. 7). In principal, a reduced seed set and seed production can be a result of low visitation rates leading to pollen limitation, as has been observed in several other plant species, especially those occurring in small and fragmented populations (Campbell 1987; Jennersten 1988; Kwak 1988; Kwak *et al.* 1991; Johnston 1991). However, a low bumblebee visitation rate does not seem to be a likely reason for the significant pollen limitation observed under natural conditions in the Oostvoorne population, since at this site each flower of *Gentiana* received at least 15 visits per day. As bumblebees appeared to be rather effective in transferring pollen and depositing it on the stigma (even after a single visit) the observed visitation rate did not seem to be limiting reproductive success. The reduced seed set and seed production following natural pollination in this population can be explained by means of the high proportion of heterospecific pollen (especially of the abundantly co-flowering *Rubus caesius*), which takes up space on the pollinator's body and may interfere with *Gentiana* pollen on the stigma (cf. stigmatic loads in the Results section; Table 1, Fig. 6; Waser 1978; Thomson *et al.* 1981; Campbell & Motten 1985; Jennersten & Nilsson 1993). This competition between conspecific and heterospecific pollen was absent in flowers which were pollinated by hand, resulting in a higher seed set. On the other hand, in the very small and isolated Castricum population very few pollinators were observed, which could perhaps be expected to lead to reduced sed set. However, in this site no difference was observed between open and hand pollinations, although the natural seed set was very low (39%). In Oostvoorne, *Rubus caesius* completely surrounded the core population of *G. cruciata*. The numerous flowers of this cornucopiously rewarding prostrate shrub were available to bumblebees at any time of the day. Bumblebees are generally not highly constant visitors to one species in an area and easily leave one nectar source as soon as another appears to be more rewarding (Heinrich 1976, 1979b). Therefore, it is not unlikely that bumblebee individuals which visited proportionally more *R. caesius* in the afternoon hours imported high amounts of heterospecific pollen into the *G. cruciata* population.

Considering that seed set after open-pollination was of the same level in both populations, we may conclude that there was also a certain degree of natural pollination in Castricum, although the observed visitation rate was very low. However, the situation seems more complicated in this case, as the overall reproductive potential (i.e. the initially produced number of ovules) was significantly lower than in the Oostvoorne site.

Differences in ovule numbers: a matter of resorption?

Another interesting aspect of the reproductive ecology of *G. cruciata* related to the low reproductive success through open-pollination is the number of ovules per flower. In general, both populations showed the largest initial number of ovules when the number of seeds and the seed set were highest (e.g. after hand-pollinations). A similar, rather more complicated phenomenon was also observed in the case of the congeneric species *G. pneumonanthe* (Petanidou *et al.* 1995). The authors argue that under conditions of nutrient deficiency and pollen limitation the non-fertilized ovules of *G. pneumonanthe*

are partly resorbed by the plant to nourish the developing embryos (see also Waser & Price 1991; Fenster 1991). After ripening of the fruit, the shrivelled ovules may be difficult to record and tend to lead to an underestimation of the number of aborted seeds and thus of the number of initially produced ovules as well. The observation that in the Oostvoorne population of *G. cruciata* the average number of ovules in fresh, unpollinated flowers was significantly higher than the average that was recorded (as the sum of aborted and viable seeds) in ripe fruits of all pollination treatments suggests that ovule resorption occurs in this species as well, and most strongly in the spontaneously and open-pollinated flowers.

Considering the data on visitation for *G. cruciata*, however, it is not very likely that any ovule resorption results from nutrient limitation as was argued for *G. pneumonanthe* (Petanidou *et al.* 1995). Since the phenomenon of ovule reduction was associated only to open-pollinated flowers of both the *G. cruciata* populations studied (cf. Fig. 7), we may argue that heterospecific pollen (from *Rubus caesius*) may negatively affect the development of conspecific pollen tubes, leading to a delayed and/or reduced fertilization. As a reaction to this, plants may start resorbing unfertilized ovules, so that seemingly a lower initial number appears to be present in the samples with open-pollination.

Population differences in reproductive potential

Another point of interest is the reduced reproductive potential (i.e. the initial number of ovules per flower) of the small Castricum population as compared to the (relatively) large Oostvoorne population. Although the seed set after spontaneous selfing and of open-pollinated flowers did not differ between the two populations, there was a significant difference between the populations in seed set after hand-pollinations. Moreover, the reduced number of ovules in the Castricum population was combined with a lower number of viable seeds per fruit after the different pollination treatments but spontaneous selfing.

Even after hand-pollination with an abundant quantity of pure conspecific pollen, maximal seed set was rather low in *G. cruciata* (the maximum observed was 70% for cross-pollination in Oostvoorne). A much lower seed set (*c.* 43%) has also been found in another perennial gentian species, *Gentiana newberryi* (Spira & Pollack 1986), but not in *Gentiana pneumonanthe* (*c.* 85%, Petanidou *et al.* 1995). A relatively high percentage of ovule or seed abortion is a frequently observed phenomenon in perennials, and it is argued that this results mainly from the higher genetic load of outcrossing perennials in comparison with selfing annuals (Wiens 1984; Wiens *et al.* 1987). In *G. cruciata*, such a high genetic load does not seem to be affecting the seed development stage, since seed and fruit set did not differ between selfed and outcrossed flowers (see above). Of course, the level of inbreeding in the populations may be so high that there is not such a large genetical difference between self and cross pollen. Indeed, the level of allozyme variation in both populations of *G. cruciata* is extremely low, as there was no variation for 15 assayed loci (C. Hermans, unpublished data). Resource limitation may be another factor reducing seed set, although it was thought to be of minor importance in a comparison of reproductive success between annuals and perennials (Wiens 1984; Wiens *et al.* 1987). For *G. newberryi*, it was argued that the low temperatures in its late flowering season limit fruit and seed set (Spira & Pollack 1986). For *G. cruciata* however, flowering occurs in the middle of the vegetative season in the dune area. It might still be possible,

however, that other kinds of environmental stress cause a reduction in reproductive potential in this species. In the perennial *Gentiana pneumonanthe*, reduced numbers of ovules, low seed set, and reduced seed weight have all been observed in small populations (Oostermeijer *et al.* 1992, 1994b). In this species, the first two may be explained by pollen limitation, and/or resorption of unfertilized ovules (Petanidou *et al.* 1995). The observed reduction in seed weight, however, was explained by environmental stress on the remaining maternal plants in small populations (Oostermeijer *et al.* 1994b). On the other hand, flower production of the offspring of small populations was reduced by inbreeding (Oostermeijer *et al.* 1994b), so it may be expected that the initial number of ovules produced per flower is also subject to inbreeding depression.

These results may help to explain the reduced reproductive potential in the smaller *G. cruciata* population. Either poor site quality or inbreeding (or combination of both) may have reduced the vitality of the maternal plants in the Castricum population, which may also influence the pollen viability of the growing plants (Young & Stanton 1990). Indeed, there appear to be some differences in the level of environmental stress between both sites. First, the Castricum population is situated at a more exposed dune ridge, and may therefore be more strongly affected by drought and salt spray. Secondly, the plants in Castricum are often completely stripped of leaves and flowers by rabbits, especially in years when the latter are numerous. This severe grazing, which in some years reduces the seed production in Castricum almost to zero, may also have lowered the vitality of the plants in this population. The influence of rabbit grazing is practically absent from the Oostvoorne population, and may therefore be an important difference between the two sites.

The differences in the recent inbreeding history of the study populations are difficult to determine. Both the Castricum and the Oostvoorne population are of rather recent origin and apparently still have a low genetic variation as a result of the founder event. It seems therefore unlikely that inbreeding has caused the observed difference in reproductive potential between both populations. However, since the Oostvoorne population may have been founded with seeds from a botanical garden, it might also have a higher variation at reproductive-trait (but not at allozyme) loci resulting from this. Since there is generally a strong relationship between allozyme heterozygosity and fitness (Ellstrand & Elam 1993), this is also not very likely.

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