

Reproductive system of the critically endangered taxon *Gentianella praecox* subsp. *bohemica*

Reprodukční systém kriticky ohroženého taxonu *Gentianella praecox* subsp. *bohemica*

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Gentianella praecox subsp. *bohemica* is a critically endangered taxon endemic to the Bohemian Massif and very dependent on appropriate management. We found that it is also very dependent on pollinator services, as the probability of autonomous selfing is low. Both hand-selfing and outcrossing resulted in similar seed production. Three out of four of the populations studied appeared to be pollen limited. The most frequent visitors and effective pollinators were bumblebees. However, we found that the different species of bumblebee differed greatly in their efficiency as pollinators due to differences in their behaviour. In contrast to other bumblebees, *Bombus wurflenii*, which nearly always robbed nectar through a hole in the corolla tube and only accidentally touched the reproductive organs, was an inefficient pollinator. Other important pollinators of *G. praecox* subsp. *bohemica* were honeybees (in lowland areas) and hoverflies. This study indicates that management for conservation should focus not only on creating gaps to improve the germination of *G. praecox* subsp. *bohemica* and reduce competition from other species, but also on improving conditions for pollinators. Hand pollination may be necessary to increase survival in populations that are close to extinction.

Key words: *Bombus wurflenii*, breeding system, *Gentianella praecox* subsp. *bohemica*, pollen limitation, pollinator efficiency

Introduction

The drastic reduction or degradation of European semi-natural grasslands in the 20th century led to a decrease in the number and size of populations of many species of plants. As a consequence, many of the plants in these grasslands became threatened (Poschlod & Wallis De Vries 2002). The most strongly threatened plants often share traits, which are disadvantageous for life in a modern landscape. Threatened grassland species are often small, have a short life span (are annual or biennial), depend on sexual reproduction and rarely reproduce clonally. Because of their small size these plants are not able to compete with large species, which profit from eutrophication and the absence of traditional management (Janeček et al. 2013, Májeková et al. 2016). A short life span and low tendency

to reproduce clonally result in low persistence in fragmented landscapes (Farnsworth 2007, Lindborg 2007). Dependency on sexual production seems to be particularly challenging for insect-pollinated plants, because changes in the 20th century not only affected plants but also their mutualistic partners. Because some groups of pollinators, as well as perennial plants, respond slowly to habitat changes (Bommarco et al. 2014), the decrease in pollinator abundance and consequent pollination crisis was realized only in the last few decades, when it was shown that the abundance of most important pollinator groups had declined dramatically across Europe (Kearns et al. 1998, Biesmeijer et al. 2006, Gómez et al. 2010). A decrease is reported for solitary bees, hoverflies (Biesmeijer et al. 2006), bumblebees (Goulson 2003) and butterflies (Brückmann et al. 2010).

The degree to which a plant will be negatively affected by a lack of pollinators is determined by the degree to which it depends on a few pollinators and its breeding system. Plants pollinated by a narrow range of pollinators, which cannot be substituted by others, are the most negatively affected. Self-compatible species, which are capable of autonomous selfing, have a relative advantage. However, increased selfing rates due to a lack of pollinators may result in inbreeding depression (Fischer & Matthies 1997, Baskin & Baskin 2015, García-Dorado 2017) and can have detrimental long-term evolutionary consequences (Wright et al. 2013). Many species prevent the negative effect of selfing by the separation of stigmas and anthers in space (herkogamy) or time (dichogamy; Richards 1986). However, in the absence of pollinators some species of plants are able to switch their breeding system from outbreeding to self-fertilization within a few generations (Roels & Kelly 2011). Because of the pollination crisis we need to improve our knowledge of the breeding system of threatened plants and their pollinators and take this information into consideration in conservation plans.

We studied the pollination ecology of the critically endangered grassland taxon *Gentianella praecox* subsp. *bohemica*. This subspecies is endemic to the Bohemian Massif and marked as critically endangered in all national or regional Red Lists within its area of distribution; Germany (Schnittler & Ludwig 1996), Austria (Niklfeld 1999), Poland (Kaćki et al. 2003) and the Czech Republic (Grulich & Chobot 2017). This plant has the above mentioned traits, which are typical for threatened grassland species. *Gentianella praecox* subsp. *bohemica* is a competitively weak, biennial, non-clonal plant dependent on sexual reproduction. Its populations depend on regular disturbance through management. Management practices have so far focused on improving the germination and growth of this taxon (Brabec 2010, Brabec et al. 2011), while very little is known about its pollinators, their effectivity and the possibility that the availability of pollen for reproduction is limited.

Studies on the breeding system of *G. praecox* subsp. *bohemica* indicate that reproduction in this plant strongly depends on visits by suitable pollinators, as autonomous selfing hardly ever occurs (Reitschläger 2000, Klaudivová 2003, Plenk et al. 2016). Gödel et al. (2010, cited in Königer et al. 2012) report that *G. praecox* subsp. *bohemica* is visited most frequently by *Apis mellifera*, *Bombus* spp. and less frequently by *Lepidoptera*, *Syrphidae* and *Muscidae*, while Plenk et al. (2016) report seeing bumblebees, honeybees, hoverflies and one species of moth entering the funnel-shaped flowers in a population in Austria. However, it is not known whether these visitors are effective pollinators. There are also no studies on possible pollen limitation. In the closely related species *Gentianella lutescens* insects belonging to the family Apoidea (*Megabombus* sp., *Parabombus* sp.,

A. mellifera and *Lassioglossum* sp.) are identified as pollinators based on their behaviour and body traits (Krupa & Jůzová 2008). In related species, pollen limitation is reported in populations of *Gentianella germanica* (Fischer & Matthies 1997, 1998, Luijten et al. 1998) and *Gentianopsis ciliata* (Oostermeijer et al. 2002, Kéry & Matthies 2004). In *Gentianella campestris*, a species capable of autonomous selfing, pollen limitation of reproduction is reported in some populations but not in others (Lennartsson et al. 2000, Lennartsson 2002).

In this study we address the following questions about the reproductive system of *G. praecox* subsp. *bohemica* and its interactions with pollinators belonging to the orders *Hymenoptera* and *Diptera* in four populations in the Czech Republic: (i) Does reproduction of this subspecies depend on pollinators? (ii) Is there pollen limitation at the sites studied? (iii) What are the main pollinators of this species and how efficient are they?

Methods

Species ecology

Gentianella praecox subsp. *bohemica* is listed as critically endangered in the Czech Republic Red List, category C1 (Grulich & Chobot 2017). It has become endangered because of habitat loss within the whole of its small distribution range, which is mainly restricted to the Czech Republic and a few adjacent areas in neighbouring countries. This taxon was first recognized as *Gentianella bohemica* by Skalický (Skalický 1969) and reclassified later to *Gentianella praecox* subsp. *bohemica* by Holub (1998) and so many populations may have disappeared before reliable documentation became available. However, there are still more than 650 well-documented historic populations, which contrasts strongly with only 111 populations recently recorded within the whole area of distribution (Brabec 2010). This species is a strict biennial, which means dependence on pollination for successful reproduction. Plants germinate and develop leaf rosettes in early spring in the first year and survive as buds over winter. In the second year the flowering stalk is formed and blooms from late August to the second half of November, followed by seed set. Part of these seeds then form a long-lived seed bank and are able to germinate even after eight years (Zahradníková & Harčariková 2007). The seeds need to undergo cold stratification before they are able to germinate (Zahradníková & Harčariková 2007). The appropriate management includes careful raking to disintegrate grass tussocks, remove mosses, and mowing or grazing by sheep and goats at a time when damage to flowering plants is minimal (Brabec 2010). This species is able to grow in many different types of grassland including *Violion*, *Nardion*, *Bromion*, *Arrhenatherion* and *Molinion* (Brabec 2010).

Study sites

We chose four populations in the Czech Republic for our studies (Electronic Appendix 1). One small population is situated near Horní Albeřice in the Krkonoše Mountains at subspecies distribution border and at the same time in the highest altitude at which the subspecies currently grows (Albeřice – 840 m a.s.l.). At the other three sites there are the largest populations with hundreds of flowering individuals or even a few thousand in

good years. All are protected in nature reserves. At the Polish border in the Orlické hory Mts there is a population growing in a *Violion* community at a high altitude (Hořečky – 820 m a.s.l.). The third population is the most isolated and in the central part of its distribution (Hroby – 500 m a.s.l.) and like the last one studied (Chvalšiny – 570 m a.s.l.) grows at lower altitudes. The management recommended by the Conservation Action Plan (Brabec 2010) is applied at all these sites.

Breeding system

The effect of different types of pollination on seed set was studied at two sites (Hroby and Chvalšiny) during the flowering season in 2016. We chose 12 similar sized plants with at least four flowering buds and covered them with mesh wire to remove the influence of individual plant vitality on the experimental treatments. To study the effect of different pollination modes on reproduction we subjected each plant to the following treatments. From two flowers we removed the anthers before blooming to prevent self-pollination. The first flower we used to simulate cross-pollination by transferring pollen from a plant at least 1 m away, the second flower was not pollinated to test for apomixis (“no pollen” treatment). The third flower was hand-pollinated by pollen from the same plant (hand-selfing) and the fourth flower was not treated in order to allow autonomous selfing. The hand-pollinated flowers were pollinated twice on two consecutive days using pollen from plants more than 1 m away. The mesh wire excluding pollinators was removed after the flowers had faded and the flowers enclosed in small mesh bags in order to prevent the loss of seeds. The ripe fruits were collected and the number of well-developed seeds counted as a measure of reproductive success. Wrinkled, small black seeds were also counted to get the percentage of well-developed seeds that had started to develop but had aborted.

Pollen limitation

We marked two open flowers in the same flowering phase of 30 plants in each of the three large study populations and of eight plants in the small mountain population (Albeřice) in order to determine whether reproduction in 2016 was limited by the availability of pollen. One flower was left to be naturally pollinated, the other was supplied with additional pollen from a plant at least 1 m away, twice on two consecutive days. After the flowers wilted they were enclosed in mesh bags to prevent the loss of seeds.

Pollinator spectrum

We recorded visitors during four days (two in 2016 and two in 2017) at each site using 7–9 portable video systems consisting of VIVOTEK (IB8367-T) or MILESIGHT (MS-C2962-FPB-IR60m) cameras. In total 393 clearly visible flowers on 122 plants were recorded during 1193 hours. During video processing we registered not only the number of approaches by insects but also how often anthers and stigmas were touched by the insects. We distinguished three groups within the *Apiidae* (*A. mellifera*, *Bombus wurflenii* and other *Bombus* spec.) and two within the *Syrphidae* (*Eristalinii* and *Syrphinii*), which differ in their behaviour.

Pollinator effectiveness

We established an experiment to determine how many seeds developed after one visit of a potential pollinator at two sites, Hořečky and Hroby, in 2017. We marked flower buds before first opening and put the whole plant under a mesh cover to prevent pollinators visiting the virgin flowers. When these flowers opened, we uncovered the plant and waited for pollinators. After a visit we caught the pollinator and stored it for further determination. Then we removed the anthers of the visited flower and enclosed the flower in a mesh bag to prevent pollinators reaching the flower and the loss of seeds. Flowers not visited by pollinators but manipulated in the same way were used as a control. After seed ripening well developed seeds were counted.

Statistics

The data from the breeding system, pollen limitation and pollinator efficiency experiments contained many zeros, i.e. no seeds developed, which is a violation of the normality assumption, so we analyzed the results using non-parametric permutation methods in the PERMANOVA program included in the software PRIMER (Anderson et al. 2008). Results of pairwise comparisons correspond to parametric two-tailed t-tests but are derived from pseudo F-values provided by PERMANOVA and P-values of these tests are obtained using permutations (Anderson et al. 2008). We used Type III sum of squares, thus every term in the model was fitted only after taking into account all other terms in the full model. The effects of site and year on the composition of the community of visitors was tested using permutation multivariate analysis of variance (PERMANOVA, Anderson 2017) in the PERMANOVA program included in PRIMER. In these analyses the rows (samples) represented individual plants, columns (species) individual visitors and values were the frequencies with which the visitors arrived. To explore if the results of these analyses are caused by total abundance and/or by proportions of visitors to individual plants we performed two tests where the samples (individual observed plants) were standardized by total of visitor arrival frequencies or not. Standardization was done by dividing the frequency of arrival of each species of insect at a flower by the total of all the frequencies for all the species that visited the flower. When the samples were not standardized the test reflects both the total abundance of visitors to a plant and proportion of visitors. In contrast, when samples (individual plants) were standardized, the analysis reflects only the proportional abundance of individual visitors to a plant and not the total abundance. To visualize the similarity among visitor communities we conducted the principal component analysis (PCA) of non-standardized data using the software Canoco for Windows 4.5 (Lepš & Šmilauer 2003). In this analysis communities of visitors to individual plants (samples) were organized in multivariate space.

Results

Breeding system

In the breeding experiment the number of seeds per capsule differed between the treatments and some of the effects differed between the two sites (Table 1, Fig. 1). At both sites, seed production after outcrossing was similar to that after hand-selfing. Flowers,

Table 1. – Results of permutational ANOVAs of the effect of various pollination treatments on the number of seeds per fruit of plants of *Gentianella praecox* subsp. *bohemica* at two sites in the Czech Republic. Plants were nested within sites. Significant differences are shown in bold.

	Effect	d.f.	F _{perm}	P _{perm}
Site	fixed	1	2.98	0.107
Plant (Site)	random	18	1.34	0.192
Treatment	fixed	3	28.31	0.001
Site × Treatment	fixed	3	3.10	0.029

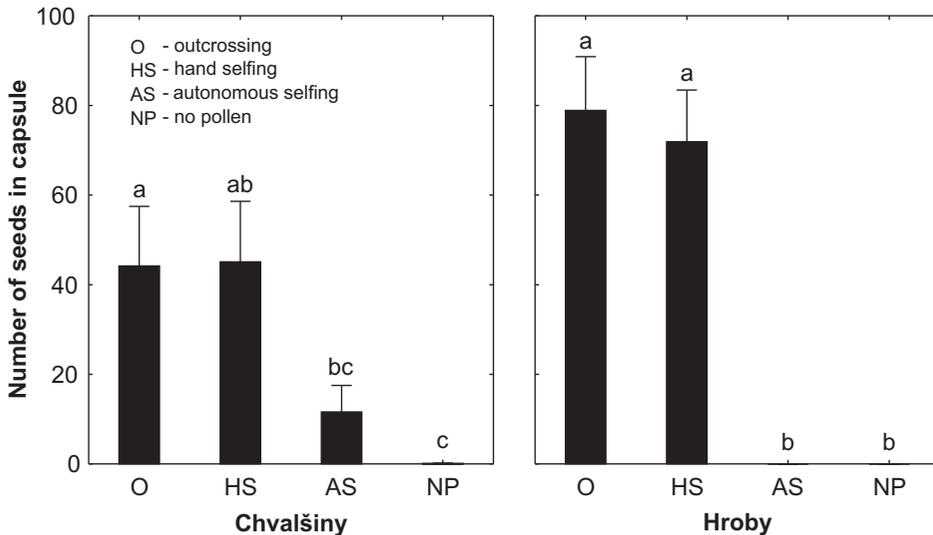


Fig. 1. – The effect of the different pollination treatments on number of seeds per fruit of plants of *Gentianella praecox* subsp. *bohemica* at two sites (Chvalšiny and Hroby). Flowers were either outcrossed, selfed using pollen from the same plant, left to self autonomously, or emasculated to test for apomixis. Means + 1 SE are shown. Different letters indicate significant differences between treatments.

whose anthers were removed did not produce seed, indicating that apomixis did not occur. At Chvalšiny, autonomous selfing produced seeds, but far fewer than outcrossing and hand-selfing, while at Hroby autonomous selfing was unsuccessful as self-pollinated flowers did not produce any seeds. To analyse if different pollination treatments influence seed abortion during development, we also analysed the percentage of well-developed seeds relative to the total number of seeds. The treatments “no pollen” and autonomous selfing were excluded, because only a few capsules contained seed. We found no difference between outcrossing and hand-selfing (Electronic Appendix 2).

Pollen limitation of reproduction

The effect of supplementary hand-pollination on seed production per fruit varied among the four populations studied (Table 2, Fig. 2). In the population at Hroby, supplementary pollination increased seed number by 15% and at Hořečky by 13%, while in the other two populations there was no clear effect (Fig. 2).

Table 2. – Results of a mixed permutational ANOVA of the effect of supplementary pollination and site on the number of seeds per fruit of plants of *Gentianella praecox* subsp. *bohemica*. Plants were nested within sites. Significant differences are shown in bold.

	Effect	d.f.	F _{perm}	P _{perm}
Site	fixed	3	1.23	0.325
Plant (Site)	random	91	2.23	0.001
Treatment	fixed	1	4.55	0.034
Site × Treatment	fixed	3	2.34	0.078

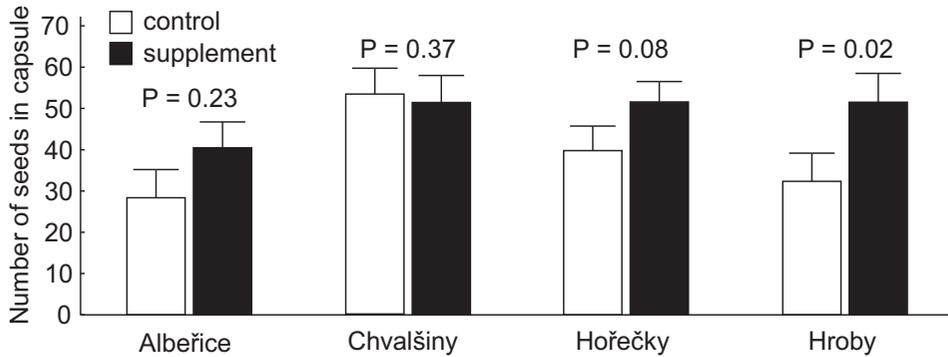


Fig. 2. – The effect of supplementary pollination on the number of seeds per fruit of plants of *Gentianella praecox* subsp. *bohemica* in four populations. Means + 1 SE are shown.

Visitors, visitation rates and behaviour

Using the portable video system, visits to 393 flowers during 1193 hours were recorded and 3056 approaches were observed. The spectrum of floral visitors differed among study sites, years and individual days (Table 3). The results for standardized and unstandardized data were similar. However, while the effect of year was significant for non-standardized data it was non-significant for standardized data, indicating differences in species abundance rather than proportional composition among years. We recorded many species of insects visiting the flowers (Electronic Appendix 3). Most of them were, however, random rather than regular visitors. The first and second PCA axes explained 21.4% and 14.1% of the total variability among the communities of visitors to individual plants (Fig. 3). The first axis separated the two mountain (Albeřice, Hořečky) from the two lowland populations (Hroby, Chvalšiny). The spectrum of visitors in the mountain populations differed from that in the lowland populations, in particular, in the presence of *B. wurflenii* and absence of *A. mellifera* (Fig. 3, 4).

Regular visitors to the flowers in all the populations were bees (*Apidae*) and hoverflies (*Syrphidae*) (Fig. 4). However, the behaviour of the various groups of visitors to flowers of *G. praecox* subsp. *bohemica* differed. *Bombus* spp., with the exception of *B. wurflenii*, almost always touched the stigma and anthers when visiting a flower. The behaviour of *B. wurflenii* was characterized by nectar robbing through holes made in the corolla tube (in

Table 3. – Effect of site, year and day on communities of floral visitors. Samples were either not standardized (abundance included) or standardized (composition of visitor communities only). Days were nested within years. Significant differences are shown in bold.

	Type	df	Not-standardized		Standardized	
			F _{perm}	P _{perm}	F _{perm}	P _{perm}
Site	fixed	3	3.48	0.039	9.73	0.012
Year	random	1	6.21	0.000	2.54	0.175
Day (Year)	random	2	2.14	0.004	2.53	0.001
Site × Year	random	3	10.84	0.000	4.16	0.000

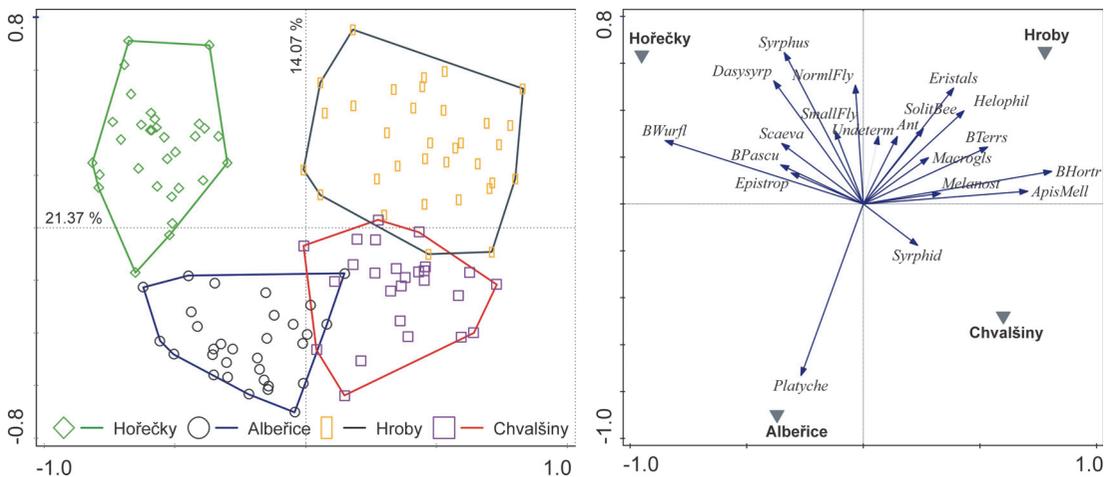


Fig. 3. – Ordination of individual plants (samples) based on the species of insects visiting *Gentianella praecox* subsp. *bohemica* in the four populations studied (left) and the association of these species of insects with individual populations (right). The arrows indicate the direction of increasing abundance of the insects relative to the four sites (triangles).

98% of its visits; Fig. 5B, C). Nevertheless, *B. wurflenii* quite often touched the reproductive organs of *G. praecox* subsp. *bohemica* with its legs (Fig. 5C, Fig. 4, see Electronic Appendix 4: video 1). The honeybee *A. mellifera* was a less frequent visitor than bumblebees even in the lowland populations. Honeybees usually delved deep into the flower to reach the nectar and touched both anthers and stigma (see Electronic Appendix 4: video 2). The two most abundant subfamilies of hoverflies, *Eristalini* and *Syrphini*, differed in their behaviour. The large hoverflies of the subfamily *Eristalini*, which were common mainly at the Hroby site, often touched anthers and/or stigmas (Fig. 5E). In contrast, the smaller hoverflies of the subfamily *Syrphini* often did not cross the paracorolla and only touched or licked the non-reproductive outer floral parts (Fig. 4, Electronic Appendix 3). However, there was one exception, hoverflies of the genus *Platycheirus*, which were the most often observed hoverflies at Albeřice, spent tens of minutes at one flower, licking and poking their heads into the flower tube.

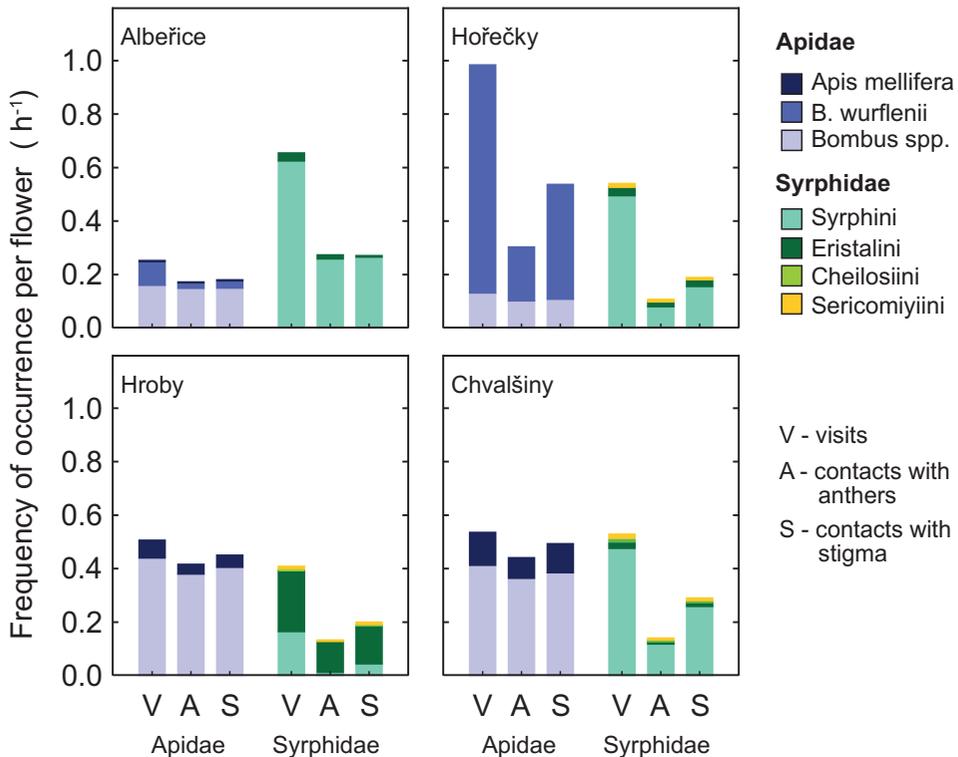


Fig. 4. – Frequency of the visits and contacts with reproductive organs of *Gentianella praecox* subsp. *bohemica* of Apidae and Syrphidae pollinators recorded in the four populations studied.

Per-visit efficiency and overall pollinator effectiveness

The effect of a single visit on seed production in the per visit efficiency experiment differed among the different groups of pollinators, both at Hroby ($F_{\text{perm}} = 4.76$; $P_{\text{perm}} = 0.012$) and Hořečky ($F_{\text{perm}} = 16.66$; $P_{\text{perm}} < 0.001$). Visits by *Bombus pascuorum* had the strongest effect on seed production in both of the populations studied (Fig. 6). At Hořečky *B. wurflenii* was a frequent visitor, but seed production after a single visit by this species was much lower than after a visit by *B. pascuorum* (Fig. 6). Small hoverflies of the subfamily *Syrphini* had the lowest mean per-visit efficiency. At Hroby, there were large hoverflies of the subfamily *Eristalini*, which were more efficient pollinators than the small hoverflies at Hořečky (Fig. 4, 6).

The overall importance of the pollinators, defined in terms of the number of seeds produced per visit times the frequency of visits was 5.92 seeds for *B. pascuorum* and 1.73 seeds for hoverflies of the subfamily *Eristalini* at Hroby. At Hořečky the importance of *B. pascuorum* was 3.28 seeds and that of *B. wurflenii* 3.46 seeds. While the overall importance of *B. pascuorum* was due to its high efficiency as a pollinator, that of *B. wurflenii* was due to its very high frequency of visits. Small hoverflies of the subfamily *Syrphini* had an overall importance of only 0.87 seeds.

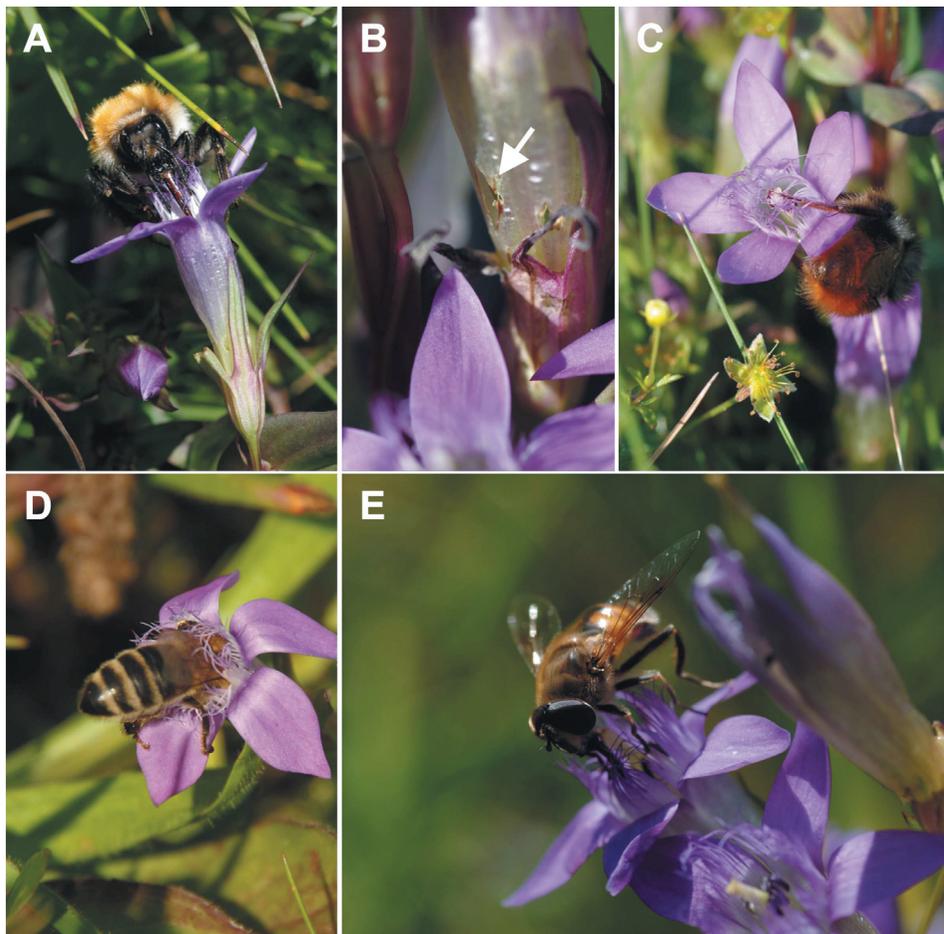


Fig. 5. – Visitors to *Gentianella praecox* subsp. *bohemica*. A – *Bombus pascuorum*. B – Hole made by *Bombus wurflenii*. C – *Bombus wurflenii* feeding on nectar via a hole in the corolla tube. However, it can also touch the reproductive organs of the plant with some parts of its body (e.g. its legs). D – *Apis mellifera* needs to climb deep into the flower to reach the nectar. E – *Eristalis* sp.

Discussion

This study shows that *G. praecox* subsp. *bohemica* has a very limited ability to autonomously self-pollinate and therefore its reproduction is strongly dependent on pollinators, in the situation when it is limited by the availability of pollen. We observed that bumblebees and hoverflies were the most common visitors and pollinators of this plant and assessed their efficiency at the sites studied.

The number of seeds that developed after hand-outcrossing and hand-selfing were similar. There are two possible reasons for this result: (i) inbreeding depression becomes manifest only at later stages in the life cycle (Barrett & Harder 1996, Fischer & Matthies 1997, Walisch et al. 2012) or (ii) the genetic load in the populations studied is so high that outbreeding results in biparental inbreeding (Uyenoyama 1986) and thus there is no

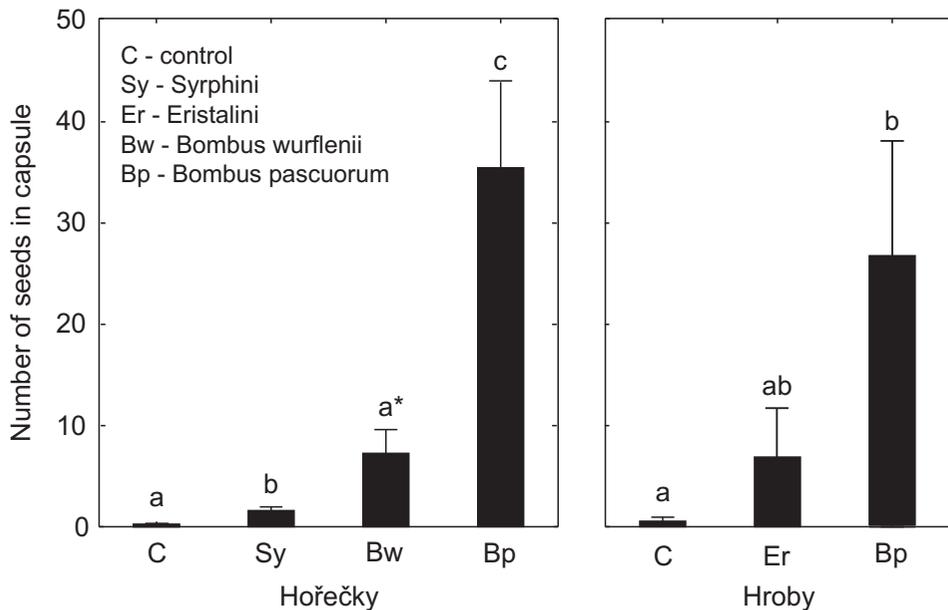


Fig. 6. – Number of seeds produced by a flower of *Gentianella praecox* subsp. *bohemica* after a single visit by various common visitors at two study sites. Means + 1 SE are shown. Different letters indicate significant differences. * Bw differs marginally from C ($P = 0.054$).

difference between self-pollination and outcrossing. This hypothesis could be tested in further studies by comparing within-population crosses and between-population crosses as in the closely related *Gentianella germanica* (Fischer & Matthies 1997). The level of biparental inbreeding could depend on the isolation and size of populations, which strongly fluctuates from year to year (Electronic Appendix 1) and thus decreases the effective size of populations. A lower performance and decrease in seed production in small populations are reported in the closely related *Gentianella germanica* (Fischer & Matthies 1998). Autonomous selfing was recorded only in one population where it was, however, much less effective than hand-outcrossing or hand-selfing. These results are consistent with those of previous studies. Klaudivová (2003) reports an average 31 ± 38 (mean \pm SD) well-developed seeds after autonomous selfing and more than double that number after hand-selfing (72 ± 37) and cross-pollination (81 ± 35). Similar experiments carried out by Reitschläger (2000) in another two Czech populations confirm lower seed production after autonomous selfing (median: 8 resp. 16) and much higher numbers after hand-selfing (median: 27 resp. 35) or cross-pollination (median: 50 resp. 34). In Austrian populations Plenk et al. (2016) report a much lower percentage of normally developed seeds after autonomous selfing than after hand-selfing or cross-pollination. However, this measure is more likely to express differences in the abortion of seed after the different pollination treatments and may indicate the degree of inbreeding depression, in contrast to the number of well-developed seed indicating the realized reproductive success. Recorded differences in the success (number of well-developed seeds) of autonomous selfing among populations could be also due to differences in flower structure. The

anthers in *G. praecox* subsp. *bohemica* are located mostly below the receptive stigma (herkogamy) and as a consequence the transport of pollen to the stigma in the absence of a pollinator is difficult. However, the degree of herkogamy could differ among populations. A study of the closely related *Gentianella germanica* (Luijten et al. 1998) indicates that the degree of spatial separation of stigma and anthers varies among populations, but there is only a negative correlation between herkogamy and autonomous selfing in one of the three populations they studied.

We found that the availability of pollen limited reproduction at three of the four sites studied, which indicates an insufficient frequency of visits by pollinators and/or low pollinator efficiency at these sites. Pollination success can be lower in small plant populations and/or in low density populations (Kunin 1997, Bernhardt et al. 2008). The characteristics of these plant populations can also influence the amount of pollen wasted because pollinators have different preferences or in the constancy of their behaviour (Fritz & Nilsson 1994). Our results indicate that pollination may be a critical factor in the survival of short-lived threatened grassland plants (e.g. Becker et al. 2011) and supports the general idea of insufficient pollination services in the current landscape (Potts et al. 2010).

We found that the spectrum of pollinators was specific for each site studied and that it differed between years. Such spatiotemporal variability seems to be the general pattern of plant-pollinator interactions as it is reported in many other recent studies (Gómez 2000, Price et al. 2005, Vlašánková et al. 2017). The spectrum of pollinators recorded in the mountain populations of *G. praecox* subsp. *bohemica* (Albeřice and Hořečky) differed from that in lowland populations (Hroby and Chvalšiny) by the presence of the mountain bumblebee species *B. wurflenii*, which showed nectar-robbing behaviour and was therefore less efficient than other bumblebee species. However, the influence of nectar-robbing species on plant reproduction need not be negative, but can be neutral or even positive (Maloof & Inouye 2000) and we need further studies to evaluate the effect of this species of bumblebee on the fitness of *G. praecox* subsp. *bohemica*. Robbing bumblebees are reported to have a negative effect on plant fitness in *Ipomopsis aggregata* (Irwin & Brody 1999), neutral effect in *Mertensia paniculata* (Morris 1996) and even a positive effect in *Anthyllis vulneraria* (Navarro 2000).

We identified bumblebees, with the exception of the nectar-robbing species *B. wurflenii*, as the most efficient pollinators. This result highlights, together with those of other studies (such as Walther-Hellwig & Frankl 2000, Westphal et al. 2003, Sepp et al. 2004), the unique importance of bumblebees as pollinators in the current agricultural landscape. Because of the flower morphology of *G. praecox* subsp. *bohemica*, large and hairy pollinators like bumblebees are more efficient than smaller insects, as has also been shown for other species with large flowers such as *Dictamnus albus* (Fisogni et al. 2016). However, hoverflies did contribute significantly to pollination in *G. praecox* subsp. *bohemica*, although their per-visit efficiency was low. For example, at the Albeřice site, the frequency of visits by hoverflies exceeded that by bumblebees and thus at least partly compensated for the lower frequency of visits by the more efficient pollinators.

Our results have implications for the future conservation of *G. praecox* subsp. *bohemica*. We demonstrate that pollination is an important process in the life cycle of this endangered species and should be taken into account in the planning of management for conservation. Supplementary hand-pollination might be considered as a management

measure to enhance the reproductive success, especially in small populations that are close to extinction (Albeřice site). However, a bigger problem for endangered species growing in grassland and many other species is the serious decrease in the abundance of flying insects (Hallmann et al. 2017) and potential pollinators in the current landscape. This can be countered by increasing the heterogeneity of landscapes, by providing suitable nesting sites for insects and flowering strips as a source of food.

See www.preslia.cz for Electronic Appendices 1–4

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Souhrn

Gentianella praecox subsp. *bohemica* je kriticky ohrožený endemický taxon Českého masívu, který je značně závislý na vhodném hospodaření. Jak se ukázalo, silně závislý je také na přítomnosti opylovačů, neboť pravděpodobnost autonomního samoopylení je velice nízká. Ve třech ze čtyř studovaných populací jsme zaznamenali sníženou reprodukci v důsledku nedostatku pylu. Nejčastějšími návštěvníky a neefektivnějšími opylovači byli čmeláci. Efektivita jednotlivých druhů čmeláků jako opylovačů se však značně lišila v závislosti na jejich odlišném chování. Čmelák *Bombus wurflenii* se ukázal být v porovnání s ostatními druhy značně neefektivním opylovačem, protože téměř vždy kradl nektar otvorem prokousaným na boku květní koruny, a reprodukčních orgánů se tedy dotýkal spíše sporadicky. Dalšími důležitými opylovači *G. praecox* subsp. *bohemica* byly včely medonosné (v nížinných populacích) a pestřenky. Naše studie poukazuje na to, že kromě vytváření vhodných podmínek pro klíčení *G. praecox* subsp. *bohemica* a snížení konkurenčního tlaku ostatních druhů by se ochranná opatření měla zaměřit i na zlepšení podmínek pro opylovače. Ruční opylení může být nezbytné pro zvýšení šancí na přežití u populací, které jsou bezprostředně ohroženy vyhynutím.

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