RESEARCH PAPER

Pollen limitation and inbreeding depression in an 'old rare' bumblebee-pollinated grassland herb

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ABSTRACT

Habitat fragmentation and reduction of population size have been found to negatively affect plant reproduction in 'new rare' species that were formerly common. This has been attributed primarily to effects of increased inbreeding but also to pollen limitation. In contrast, little is known about the reproduction of 'old rare' species that are naturally restricted to small and isolated habitats and thus may have developed strategies to cope with long-term isolation and small population size. Here we study the effects of pollen source and quantity on reproduction of the 'old rare' bumblebee-pollinated herb, *Astragalus exscapus*. In two populations of this species, we tested for pollen autodeposition, inbreeding depression and outbreeding depression. Caged plants were left unpollinated or were pollinated with pollen from the same plant, from the same population or from a distant population (50 km). Additionally, we tested for pollen limitation by pollen supplementation in four populations of different size and density. In the absence of pollinators, plants did not produce seed whereas self-pollinated plants did. This indicates a self-compatible breeding system dependent on insect pollination. Both self-pollination and, in one of the two populations, cross-pollination with pollen from plants from the distant population resulted in a lower number of seeds per flower than cross-pollination with pollen from plants from the resident population, indicating inbreeding and outbreeding depression. Pollen addition enhanced fruit set and number of seeds per flower in three of the four populations, indicating pollen limitation. The degree of pollen limitation was lowest in the smallest but densest population. Our results suggest that, similar to 'new rare' plant species, also 'old rare' species may be at risk of inbreeding depression and pollen limitation.

INTRODUCTION

Habitat fragmentation and reduction of population size have been found to negatively affect plant reproduction in 'new rare' species which were formerly common (Hauser & Loeschke 1995; Ouborg & van Treuren 1995; Aguilar *et al.* 2006). This has been attributed primarily to effects of increased inbreeding but also to pollen limitation in formerly large and outcrossing populations. In contrast, little is known about the reproduction of 'old rare' species that are naturally restricted to small and isolated habitats and thus may have developed strategies to cope with long-term isolation and small population size (but see *e.g.* Mavraganis & Eckert 2001; Leimu 2004).

In general, the sensitivity of a plant species to habitat fragmentation largely depends on its breeding system, for example, the ability to produce seeds in the absence of pollinators (Aizen *et al.* 2002; Aguilar *et al.* 2006). In plant species depending on pollinators for reproduction it has been suggested that the most prominent cause of reproductive impairment in fragmented habitats and small populations may be insufficient

pollination (Aguilar *et al.* 2006). Pollen limitation can affect plant abundance and population viability and lead to selection on plant mating system and floral traits (*e.g.* Johnston 1991; Ashman *et al.* 2004). Larger populations present a larger total floral display, which may improve foraging efficiency and hence make them more attractive to pollinators (Goulson 2000). Small populations, in contrast, often suffer from pollen limitation, *i.e.* their reproduction is limited due to low pollen quantity and low pollen quality (Lennartsson 2002; Aizen & Harder 2007; Kolb 2008; Dauber *et al.* 2010). In addition to population size, the density of plants may also have consequences for pollination as it may lead either to the attraction of more pollinators (*i.e.* positive density effect; Kunin 1993) or to their dilution (*i.e.* negative density effect; Rathcke 1983).

Low pollen quantity is a consequence of low visitation rates of pollinators or may arise when single pollinators transfer little pollen (Ghazoul 2005; Knight *et al.* 2005). In contrast, poor pollen quality may be the consequence of self-pollination and crossing between close relatives (*i.e.* biparental inbreeding), either of which may lead to inbreeding

depression (Lloyd & Schoen 1992; Griffin & Eckert 2003). Negative effects of inbreeding have been found on various components of plant fitness, *e.g.* seed quantity and quality, seed germination, plant growth and seed production (Hauser & Loeschke 1995; Severns 2003). While inbreeding depression reduces the fitness of selfed offspring, outcrossed offspring will be more likely to contribute to the next generation (Herlihy & Eckert 2002; Michalski & Durka 2007). Thus, inbreeding depression helps to maintain high effective outcrossing rates despite some self-pollination.

Inbreeding depression is expected to arise particularly in plant species that were formerly common and have recently become rare, *e.g.* due to habitat fragmentation (Huenneke 1991). These 'new rare' species may have accumulated a genetic load in the form of recessive deleterious alleles during long periods of large population size and high outcrossing rates (Husband & Schemske 1996). After reduction of population size such alleles may be expressed as a result of inbreeding and may lead to reduced reproductive fitness (*e.g.* Lienert & Fischer 2004). In contrast, if populations have been small for long periods of time deleterious alleles may have been selected against (purging), and therefore increased self-pollination does not necessarily result in inbreeding depression (Lynch *et al.* 1995; Byers & Waller 1999). This pattern may therefore be expected in 'old rare' plant species; however, in small populations deleterious alleles may also be fixed due to genetic drift, increasing extinction probability (Vilas *et al.* 2006).

Pollen flow among populations increases genetic exchange and thus reduces inbreeding (Dudash & Fenster 2000). Progeny may benefit from hybridisation between populations when deleterious alleles that were fixed in the parental populations become hidden in the hybrid population as heterozygotes. Thus, between-population crosses may lead to increased seed mass and germination due to heterosis (Seltmann *et al.* 2009). Hybridisation between populations may thus contribute to a recovery from genetic drift or inbreeding depression (Keller & Waller 2002; Hufford & Mazer 2003). However, pollen flow between widely separated individuals may also have deleterious effects due to the disruption of local adaptation or allelic co-adaptation, resulting in outbreeding depression (Dudash & Fenster 2000). Outbreeding depression may occur at scales of hundreds of metres in spatially structured populations (*e.g.* Waser *et al.* 2000; Schleuning *et al.* 2010), but is most commonly found as a result of crosses between widely separated populations (*e.g.* Fischer & Matthies 1997).

Here, we study effects of pollen quantity and pollen source in the bumblebee-pollinated plant *Astragalus exscapus* growing in European dry grasslands. This species is rare and considered a relict of the Pleistocene steppe vegetation. It has survived in isolated small grassland patches on south-facing slopes since the mid-Holocene when woodland nearly completely covered the area (Walter & Straka 1970; Becker 2010).

We studied the breeding system of *A. exscapus*, performed manual self- and outcross-pollination treatments and pollen addition experiments, and analysed fruit and seed production of *A. exscapus* in four populations. We expected (i) low levels of inbreeding depression because populations may have purged their genetic load after long periods of being small and isolated; while (ii) outbreeding depression is expected because populations may have undergone genetic drift during long-term isolation; (iii) Finally, we expect populations to

suffer from pollen limitation because of the small population size of the populations studied.

MATERIALS AND METHODS

Study species

Astragalus exscapus L. (Stemless milkvetch, Fabaceae) has a disjunct distribution range and is confined to dry grasslands in Europe. Due to its conspicuity and attractiveness, as well as active mapping by local botanists, almost all populations of *A. exscapus* in the study region (Kyffhäuser Mountains, Central Germany) have been known for at least 100 years and only very few new populations have established during that time (Becker 2003), underlining the long fragmentation history of its populations. The species is a rare, long-lived (at least up to 21 years) herb, which reproduces solely *via* seeds (Becker 2003). The plants have a reduced stem, and the lemon-yellow odoriferous flowers appear in five to 400 racemes with each of four to eight flowers a few centimetres above the ground (Kienberg O., unpublished data). In the study region, plants start to flower in mid-April and finish flowering at the end of May. Flowers are *ca.* 27 × 5 × 8 mm, are homogamous (Knuth 1898) and have a valvular mechanism of pollination, *i.e.* keel and wings of the flower are folded down by pollinators, which thereby take up new pollen ventrally and deposit foreign pollen onto the stigma. Typically, *A. exscapus* is pollinated by bumblebees (*e.g.* *Bombus hortorum*, *B. pascuorum*), which gather both nectar and pollen, while *B. terrestris* has been observed to break into flowers laterally and rob nectar (Knuth 1898). After successful pollination, one fruit (pod) is produced per flower, which contains between one and 18 bean-shaped seeds (mean 3.6 ± 1.95 ± SD, n = 4220 pods) (Becker 2010). Predispersal seed predation by seed-parasitic wasps (*Eurytoma* spp.) damages parts of the seed (on average 14% ± 2.1% ± SD, n = 100 plants) (Becker 2010). As a supplementary characterisation, we assessed the pollen/ovule ratio on seven flowers from different populations by suspending the total amount of pollen per flower in 200 µl water and counting pollen grains in five 2-µl aliquots and by dissecting and counting ovules under a binocular microscope. This revealed a mean of 23,077 pollen grains per flower (14,266–34,400), 12 ovules per flower (9–13) and a mean pollen/ovule ratio of 2070 (±SE 310; 1297–3822).

The species is rare and declining, and is considered threatened in most countries throughout its total range (Becker 2003). In Germany, for example, at least 44 populations, *i.e.* 44% of all populations known in the country, have become extinct in the last 150 years (Becker 2003). In total, *A. exscapus* occurs in 55 populations in Germany, four of which were investigated here.

Study sites and populations

The study populations, 'Vatersberg' (11°02′63″ E, 51°22′34″ N, referred to as population 1 hereafter), 'Kosakenstein' (11°05′06″ E, 51°21′83″ N, population 2), 'Barbarossahöhle' (11°02′24″ E, 51°22′58″ N, population 3) and 'Nußtälchen' (11°04′73″ E, 51°22′03″ N, population 4), are located in the Kyffhäuser Mountains, Central Germany, at 160–225 m a.s.l. Annual precipitation is low (about 500 mm), summers are warm (mean temperature in July 17.6 °C) and winters relatively cold (mean temperature in January −0.8 °C), indicating

sub-continental climatic conditions (Becker 2003). The maximal distance between study populations was 3.4 km and the distance to the next population ranged from 0.26 to 0.45 km. Populations are located on south-facing slopes in sub-continental dry grasslands. In addition, populations 1 and 3 are situated partly in semi-dry grasslands (Becker 2010). The number of flowering plants, the area (minimal convex polygon around plants) and density of the populations were as follows: population 1: 355 plants on 350 m^2 (density 1.01 m^{-2}), population 2: 57 plants on 1200 m^2 (0.05 m^{-2}), population 3: 1104 plants on 2000 m^2 (0.55 m^{-2}) and population 4: 24 plants on 20 m^2 (1.20 m^{-2}).

Breeding system and effects of self- and cross-pollination

Effects of pollinator exclosure and cross-pollination were studied in populations 1 and 2. Effects of geitonogamous self-pollination were studied only in population 2 because, in population 1, some plants had too few flowers to conduct all pollination treatments. In each population, 10 adult plants were chosen at random and enclosed before flowering in nylon mesh bags (*ca.* 40 × 40 × 40 cm, mesh size = 1.5 × 1.5 mm) to exclude pollinators and seed dispersers or predators. During the 6-week pollination experiment, each of 10 flowers per plant (10 flowers × 10 plants × 2 populations) were (i) left unpollinated; (ii) self-pollinated by hand with pollen from three other flowers of the same plant (geitonogamous selfing); (iii) cross-pollinated by hand with pollen from three flowers from three other plants each 2–4 m from the acceptor plant (within-population cross); and (iv) cross-pollinated by hand with pollen from three flowers from three other plants from a population at about 50 km distance (Lake Süßer See, 11°41′10″ E, 51°29′51″ N) (between-population cross). Ten randomly chosen plants

Table 1. Effects of three pollination treatments on (A) fruit set, (B) number of seeds per flower, (C) number of seeds per fruit, (D) number of aborted seeds, and (E) seed mass in two populations of *Astragalus exscapus*. The effect of pollen addition was partitioned into three orthogonal contrasts: (i) Control *versus* hand-pollination; and (ii) self- (geitonogamous pollination) *versus* cross-pollination; and (iii) within-population cross-pollination (WPC) *versus* between-population cross-pollination (BPC). Results are from ANOVA.

source of variation	SS	df	F	P	SS	df	F	P
population	1				2			
(A) fruit set								
treatment	1.49	2	6.41	**0.005**	1.60	3	6.24	**0.001**
control *versus* hand	1.41	1	12.34	**0.002**	1.57	1	19.49	**<0.001**
self *versus* cross	–	–	–	–	0.01	1	0.03	0.870
WPC *versus* BPC	0.08	1	2.03	0.173	0.03	1	1.82	0.198
error	3.02	26			2.47	29		
total	4.51	28			4.07	32		
(B) number of seeds per flower								
treatment	36.13	3	12.84	**<0.001**	96.42	4	29.72	**<0.001**
control *versus* hand	13.69	1	11.23	**0.002**	28.23	1	21.68	**<0.001**
Self *versus* cross	–	–	–	–	7.35	1	5.99	**0.022**
WPC *versus* BPC	0.08	1	0.05	0.834	1.21	1	0.86	0.371
error	32.84	35			29.20	36		
total	68.97	38			125.62	40		
(C) number of seeds per fruit								
treatment	2.91	2	0.55	0.582	17.26	3	4.18	**0.015**
control *versus* hand	1.05	1	0.40	0.530	6.83	1	4.14	**0.050**
self *versus* cross	–	–	–	–	10.33	1	8.17	**0.009**
WPC *versus* BPC	1.86	1	0.93	0.350	0.09	1	0.56	0.817
error	65.74	25			35.79	26		
total	68.68	27			53.05	29		
(D) aborted seeds								
treatment	1.56	2	0.55	0.586	15.15	3	2.24	0.113
control *versus* hand	1.16	1	0.84	0.369	3.78	1	1.48	0.236
self *versus* cross	–	–	–	–	0.77	1	0.27	0.609
WPC *versus* BPC	0.40	1	0.30	0.593	10.60	1	4.73	**0.050**
error	32.90	23			47.31	21		
total	34.47	25			62.46	24		
(E) seed mass								
treatment	1.40	2	0.62	0.547	0.55	3	0.19	0.905
control *versus* hand	0.58	1	0.52	0.479	0.30	1	0.32	0.574
self *versus* cross	–	–	–	–	0.04	1	0.04	0.836
WPC *versus* BPC	0.82	1	0.70	0.417	0.21	1	0.21	0.658
error	20.15	18			23.76	24		
total	21.55	20			24.31	27		

Significant *P* values are in bold.

were left uncaged and untreated and used as (v) controls (open-pollination).

Pollination treatments were carried out *ante meridiem*. Pollen was obtained by removing a flower from the donor plant and inserting the exposed anthers carefully into the donor flowers to take up the pollen. Removed flowers were stored in a plastic box and used for hand-pollinations within 2 h. Target flowers were not emasculated in order not to disturb flowering. Hand-pollinated flowers were marked with coloured wool fibres. After approximately 4 weeks, when all flowers had withered and seeds were mature, inflorescences were collected to determine the initial number of treated flowers, the number of developed fruits, the number of developed seeds per fruit, the number of aborted seeds (the latter using a microscope) and seed mass. We calculated average values per plant of (i) fruit set (number of fruits/number of flowers); (ii) number of seeds per fruit (number of seeds/number of fruits); and (iii) number of seeds per flower (number of seeds/number of flowers). While number of seeds per fruit reflects quality of deposited pollen, the number of seeds per flower reflects both quality and quantity of deposited pollen, and thus is a more appropriate measure for total reproductive success.

Effects of pollen supplementation

Effects of pollen quantity were studied in all four populations by supplemental hand-pollination. In each population 10 plants were selected randomly and 10 flowers per plant were hand-pollinated with mixed pollen from three donor plants at a distance of 2–4 m from the acceptor plants. To assure a mixed pollen source, we used three flowers (one flower each from three donor plants) to pollinate three to four acceptor flowers; thus a total of nine donor flowers were used to pollinate the 10 acceptor flowers. Pollination was performed as described above, but plants were not caged in order to ensure open-pollination. Ten additional flowers per plant were left untreated and used as a control. Fruit set and number of seeds per flower were determined as above. The data from the pollen supplementation experiment were used to calculate a pollen limitation index: $PL = 1-(S_o/S_s)$, where S_o and S_s are fruit set or number of seeds per flower with open-pollination or supplemental pollination, respectively (Larson & Barrett 2000). Thus, PL_{fruit} refers to pollen limitation based on fruit set and PL_{seed} refers to number of seeds per flower. Negative values indicating a higher number of seeds per flower in open than in supplemented flowers were observed in some plants in population 4. This population already had high fruit set and a high seed number per flowers without pollen supplementation, making detection of a further increase less likely. These values were set to zero, as also done by Larson & Barrett (2000) and Jakobsson *et al.* (2009). PL was first calculated at the individual plant level and then averaged within populations.

Statistical analysis

We checked for normal distribution and log-transformed fruit set prior to the analyses to achieve the desired distribution. Effects of treatments (ii) to (v) were analysed with ANOVA and mean differences were tested with a Tukey *post hoc* test or as orthogonal *a priori* contrasts (control *versus* hand, self *versus* cross, and within-population cross *versus* between-population

cross). Effects of pollen supplementation and the interaction between population and treatment were analysed with ANOVA using both treatment and population as fixed factors. All statistical analyses were performed with SPSS 15.0 (SPSS Inc. 2005, Chicago, IL, USA).

RESULTS

Breeding system and effects of self- and cross-pollination

Bagged untreated flowers did not produce fruits, indicating that autonomous self-pollination does not occur. Fruit set and seed production in selfed flowers indicated self-compatibility.
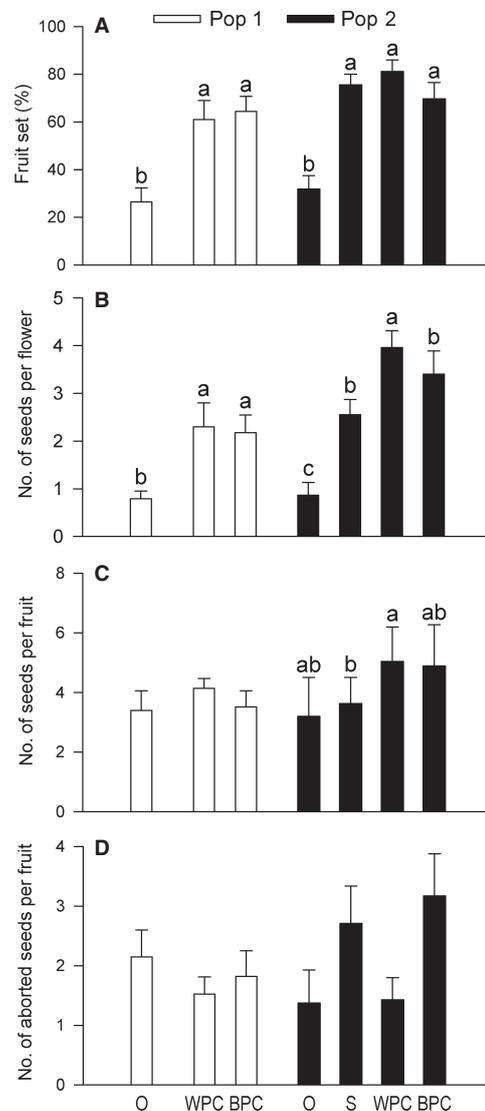
Fig. 1. (A) Fruit set, (B) number of seeds per flower, (C) number of seeds per fruit, and (D) aborted seeds in two populations of *Astragalus exscapus* after open-pollination (O), self-pollination (S) (geitonogamous pollination) and cross-pollination from the resident (WPC – within-population cross) and another population (BPC – between-population cross). Mean and ±1 SE is given. Significant differences between treatments within populations are indicated by different letters (Tukey *post hoc* test, P > 0.05).

Hand-pollination led to a more than two-fold increase in fruit production and more than three-fold increase in seeds per flower compared to open-pollination, irrespective of pollen source, indicating pollen limitation in both populations (Table 1A and B, Fig. 1A and B). There were no differences in fruit set between flowers that were selfed or crossed with pollen from the local population (within-population cross) compared to those crossed with the distant population (between-population cross). In population 2 the number of seeds per flower was significantly (31%) lower in selfed flowers than in outcrossed flowers (Table 1B, Fig. 1B). In the same population, the number of seeds per flower was significantly (14%) lower, and the number of aborted seeds was higher, in the between-population cross than in the within-population cross (Table 1B and D, Fig. 1B and D). In population 1, between- and within-population crosses resulted in the same number of seeds per flower (Fig. 1B). In contrast to the number of seeds per flower, the number of seeds per fruit did not change much between open-pollination and hand-pollination treatment (Fig. 1C), indicating that pollen quantity rather than pollen quality was limiting. Seed mass did not differ between treatments (Table 1E).

Table 2. Effects of supplemental hand-pollination treatment and population on (A) fruit set, (B) number of seeds per flower, (C) number of seeds per fruit, (D) number of aborted seeds, and (E) seed mass in four populations of *Astragalus exscapus*.

	SS	df	F	P
(A) fruit set				
population	2.05	3	5.27	**0.003**
treatment	1.89	1	14.55	**<0.001**
population × treatment	1.03	3	2.65	0.057
error	7.78	60		
total	12.74	67		
(B) number of seeds per flower				
population	12.05	3	4.13	**0.010**
treatment	25.39	1	26.12	**<0.001**
population × treatment	4.31	3	1.48	0.230
error	58.33	60		
total	100.08	67		
(C) number of seeds per fruit				
population	4.54	3	0.55	0.651
treatment	0.23	1	0.08	0.772
population × treatment	15.68	3	1.89	0.141
error	154.58	56		
total	175.03	63		
(D) aborted seeds				
population	27.02	3	5.39	**0.003**
treatment	0.01	1	0.01	0.981
population × treatment	7.88	3	1.57	0.208
error	81.87	49		
total	116.77	56		
(E) seed mass				
population	7.29	3	2.23	0.100
treatment	1.71	1	1.57	0.218
population × treatment	5.61	3	1.72	0.179
error	42.47	39		
total	57.08	46		

Significant *P* values are in bold.

Effects of pollen supplementation

Supplemental pollination had a strong effect on fruit set and number of seeds per flower (Table 2, Fig. 2). Both significantly increased in three of the four populations, but not in the smallest and most dense population (population 4), which showed high fruit set and number of seeds per flower even in the absence of supplemental pollen. Thus, the degree of pollen limitation was strong for both fruit set (mean $PL_{fruit} = 0.44$, across all populations) and for the number of seeds per flower (mean $PL_{seed} = 0.49$, across all populations). Pollen limitation was highest in the three larger populations,
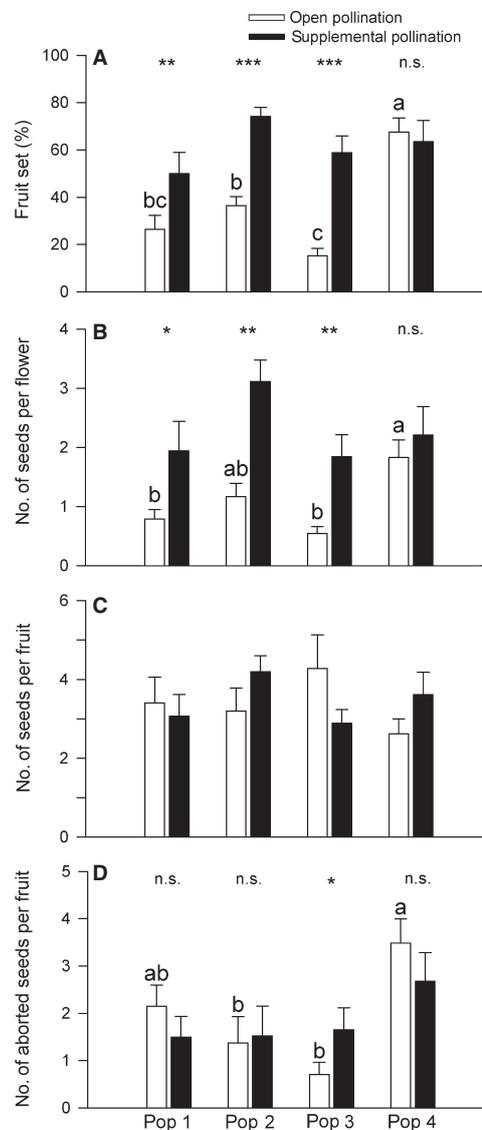
Fig. 2. (A) Fruit set, (B) number of seeds per fruit, and (D) number of aborted seeds in four populations of *Astragalus exscapus* after open-pollination and supplemental hand-pollination. Mean and 1 SE are given. Significant differences between treatments within populations are indicated by asterisks: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, n.s., not significant at $P < 0.05$; differences between populations within treatments (open and supplemental hand-pollination, respectively) are indicated by different letters above the respective treatments (ANOVA, $P < 0.05$). Statistics are given for significant differences only.
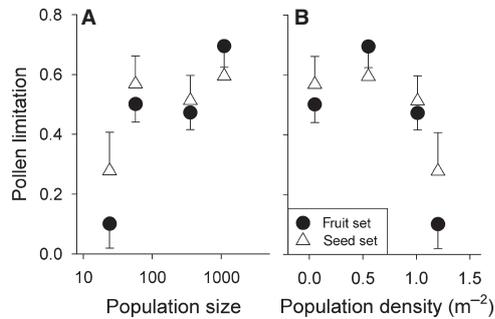
Fig. 3. Pollen limitation of fruit set and pollen limitation of number of seeds per flower as a function of (A) population size and (B) density. Mean and ±1 SE is given.

which had a lower density, and was lowest in the smallest population, which had the highest density (Fig. 3). However, this effect was significant only for PL_{fruit} ($F_{3,28} = 11.9$, $P < 0.001$) but less pronounced for the number of seeds per flower for PL_{seed} ($F_{3,28} = 1.70$, $P = 0.190$), as indicated by ANOVA and *post hoc* tests. The number of seeds per fruit and seed mass showed no treatment effect and did not differ between populations (Table 2C, Fig. 2C). Similarly, the number of aborted seeds only changed in one population (population 2), where it increased after supplemental pollination (Fig. 2D).

DISCUSSION

Breeding system

In the absence of pollinators, *A. exscapus* did not produce seeds, whereas self-pollinated plants did. This result indicates that the species is self-compatible but reliant on pollinators. *Astragalus* is a large genus and most species have been found to be self-compatible, including both annuals (*e.g.* Gallardo *et al.* 1994) and several other long-lived species (Karron 1987, 1989; Kaye 1999; Kudo & Molau 1999). However, self-incompatibility has also been reported in *A. cicer* (Townsend 1971; but see Kirchner 1905). *A. exscapus* did not display autonomous selfing but depended on insects for pollination. Therefore, the claim for spontaneous self-pollination in *A. exscapus* in the absence of pollinators (Gams 1964) must be corrected. The lack of autonomous self pollination in *A. exscapus* is thus in line with the general observation in Fabaceae that long-lived species mostly do not produce seeds in the absence of pollinators, while most short-lived species do (Kirchner 1905).

Inserting a cotton bud into a flower of *A. exscapus* resulted in successful self-pollination. Therefore, the lack of autonomous self-pollination could not be explained through dichogamy. In addition, in *A. exscapus* the anthers are located near the stigma so that contact with pollen seems to be inevitable even when flowers are closed (Knuth 1898). In several self-incompatible legumes (*e.g. Trifolium pratense, Lotus* spp.) contact of pollen and stigma is inhibited by a pellicula on the stigma, which is perforated by pollinating insects (Heslop-Harrison & Heslop-Harrison 1983; Owens 1985), a mechanism also assumed to exist in *A. australis* var. *olympicus* (Kaye 1999). However, inspection with a light microscope revealed neither a pellicula nor other types of pollen barrier, but rather viscous exudates were found on the stigma (Becker T.,

personal observation). Thus, the efficient avoidance of pollen autodeposition in *A. exscapus* could not be fully explained.

The pollen/ovule ratio of *A. exscapus* was one order of magnitude higher than that of reportedly selfing *Astragalus* species (Gallardo *et al.* 1994) and similar to that of other perennial *Astragalus* species (cf. Rodriguez-Riano *et al.* 1999), which most probably have a mixed mating or outcrossing mating system. Thus, although P/O ratios are not direct indicators of mating system (Michalski & Durka 2009), the comparatively large P/O ratios of *A. exscapus* within the genus (Galloni *et al.* 2007) suggest a mixed mating breeding system.

Inbreeding and outbreeding depression

Hand self-pollination resulted in a lower number of seeds per flower and a higher number of aborted seeds than within-population cross-pollination. This indicates inbreeding depression at the seed development stage and suggests that the genetic load has accumulated during the evolutionary history of the species. In contrast to other 'old rare' plant species, which do not suffer from inbreeding depression (*e.g.* Leimu 2004; Rabasa *et al.* 2009), in *A. exscapus* genetic load obviously has not been purged during the phases of isolation and despite small population size in general. However, we have studied hand self-pollination in only one population, and therefore the general importance of early inbreeding depression must be verified in a future study. Inbreeding depression often becomes visible in later plant stages, *e.g.* seedling performance or reproduction of adult plants (Husband & Schemske 1996). Thus, although only a moderate (31%) reduction in number of seeds per fruit, and therefore only moderate inbreeding depression was detected, stronger inbreeding depression might occur in a later stage of the plant's life cycle. In fact, results of allozyme analyses indicate that *A. exscapus* populations are only weakly inbred at the adult stage, as evidenced by a mean inbreeding coefficient across 37 populations of $F_{IS} = 0.129$ ($P = 0.292$) (Becker 2003). Hence, although the species is self-compatible and bumblebee pollinators may partly accomplish geitonogamous selfing, inbreeding depression seems to be efficient in avoiding selfed offspring prior to adulthood.

Pollination with pollen from a distant population resulted in lower seed set and a higher number of aborted seeds in one of the two populations, suggesting outbreeding depression. However, in the other population no effect was found, indicating that outbreeding depression may be population-specific. Outbreeding depression has also been found in other plant species (*e.g.* Fischer & Matthies 1997; Waser *et al.* 2000), including those with fragmented populations (*e.g.* Mooney & McGraw 2007). However, similar to inbreeding depression, outbreeding depression may be stronger at later stages or in later generations, because recombination disrupts the original parental gene combinations and exposes incompatibilities involving recessive alleles (Edmands & Timmerman 2003).

Pollen limitation in relation to population size

Our results show that reproduction of *A. exscapus* strongly depends on bumblebees for pollination. Moreover, pollen addition enhanced fruit set and number of seeds per flower in three of four populations, indicating pollen limitation in most populations. Pollen limitation is generally thought to be stron-

ger in small or less dense populations than in large or dense populations (Knight *et al.* 2005; for *Astragalus* spp., see also Crone & Lesica 2006). In the studied populations, population size and population density were negatively correlated. Thus, we cannot disentangle these two factors; however, the degree of pollen limitation was lowest in the smallest population, which had the highest plant density. This suggests that high population density may be able to compensate for small population size. Positive density-dependence of pollination has frequently been reported (Cheptou & Avendano 2006; Zorn-Arnold & Howe 2007; Jakobsson *et al.* 2009). With increasing plant density the pollinators may be more efficient due to increased supply of nectar and pollen. Therefore they may visit either a greater proportion of plants or a smaller proportion of flowers per visited plant (Jakobsson *et al.* 2009). The study species has shiny yellow flowers, which, however, are exposed close to the soil surface. Thus, higher population density may particularly increase population conspicuousness, thereby increasing pollinator visitation rates. However, as the density and population size effect was only observed in one population, other population-specific effects cannot be excluded as the underlying cause, *e.g.* the identity or density of pollinators.

In conclusion, our study suggests that 'old rare' species persisting for a long time in isolated and small populations can maintain a predominantly outcrossing mating system and do not necessarily evolve towards autogamy as a consequence of strong pollen limitation (Ashman *et al.* 2004). Thus they may be at risk of inbreeding due to accumulated genetic load, similar to previously common but now rare species. Although *A. exscapus* attracts pollinators with a large floral display of odoriferous flowers and earlier phenological flowering time than most other plants in dry grasslands, pollen limitation was evident in most populations. Therefore, even 'old rare' species such as *A. exscapus* may benefit from management regimes that try to enhance both habitat quality and pollination service.

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