Experimental inbreeding reduces seed production and germination independent of fragmentation of populations of *Swertia perennis*

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Abstract

We studied effects of inbreeding on the early fitness measures seed production and germination in *Swertia perennis* L. (Gentianaceae), a locally abundant specialist of fen grasslands, which have been greatly fragmented in Switzerland. We further tested whether such effects differed between populations of different size and isolation. We studied effects of free pollination, hand-outcrossing and hand-selfing on 160 plants in 16 populations in large, in small barely isolated, and in small isolated habitat islands. We found a distinct gradient in experimental outcomes from free pollination to hand-outcrossing to hand-selfing. Compared with free pollination, selfing reduced the number of seeds per capsule by 22% (P < 0.01), the seed/ovule ratio by 26% (P < 0.001), seed mass by 25% (P < 0.001) and germination percentage by 23% (P < 0.05). The outcome of hand-outcrossing was intermediate for all traits. The reduction in early fitness components after selfing is most likely due to inbreeding depression. Higher seed production and germination after free pollination than after hand-outcrossing may be due to a larger number of pollen donors involved in free pollination or due to higher quality of free pollen because of lower relatedness of parent plants. Moreover, the fact that seed production was highest after free pollination indicates that *S. perennis* is not pollen limited. Pollination treatment effects did not differ between different island types. Hence, there are no indications that early inbreeding depression was purged from more strongly fragmented populations.

Wir untersuchten die Auswirkungen von Inzucht auf die frühen Fitnessmerkmale Samenproduktion und Keimung bei der lokal häufigen *Swertia perennis* L. (Gentianaceae), einer Pflanze der in der Schweiz stark fragmentierten Flachmoore. Um zu untersuchen, ob sich solche Auswirkungen in verschieden großen und unterschiedlich isolierten Populationen unterscheiden, verglichen wir die Auswirkungen von freier Bestäubung, Handfremdbestäubung und Handselbstbestäubung für 160 Pflanzen in 16 Populationen in großen, in kleinen wenig isolierten und in kleinen isolierten Habitatinseln. Wir fanden einen ausgeprägten Gradienten von freier Bestäubung über Fremdbestäubung von Hand zu Handselbstbestäubung: Im Vergleich zu freier Bestäubung war nach Selbstbestäubung die Anzahl Samen pro Kapsel um 22% (P < 0.01), der Samenansatz um 26% (P < 0.001), die Samenmasse um 25% (P < 0.001) und der Keimungserfolg um 23% (P < 0.05) herabgesetzt. Die Resultate der Handfremdbestäubung waren für alle Maße intermediär. Die Herabsetzung von Merkmalen früher Fitness von *S. perennis* nach Selbstbestäubung ist höchstwahrscheinlich eine Folge von Inzuchtdepression. Der im Vergleich zu Handfremdbestäubung nach freier Bestäubung höhere Fortpflanzungserfolg und die höhere Keimung könnten durch eine größere Anzahl zu freier Bestäubung beitragende Pollenspender oder durch geringere Verwandtschaft der in freier

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Bestäubung beteiligten Elternpflanzen bedingt sein. Dass die Samenproduktion nach freier Bestäubung am höchsten war, deutet auch darauf hin, dass *S. perennis* nicht pollenlimitiert ist. Die Effekte der Bestäubungsbehandlungen unterschieden sich nicht zwischen Populationen der verschiedenen Habitatinseltypen. Es gibt also keinen Hinweis darauf, dass frühe Inzuchtdepression aus kleinen und stärker isolierten Habitatinseln ausgemerzt wurde.

Key words: conservation biology – Gentianaceae – habitat fragmentation – inbreeding depression – geographic isolation – pollination experiments – population size – reproduction – wetlands

Introduction

Recent habitat fragmentation may severely change levels of inbreeding in plant populations. Reduced size and increased isolation of populations increases the importance of genetic drift, which can increase average relatedness of plants within populations (Ellstrand & Elam 1993). Moreover, pollinator service can be changed by fragmentation, which may lead to pollen limitation and to increased selfing levels and relatedness of cross-pollinating plants (Heinrich 1979, Kunin 1993, Groom 1998).

Increased inbreeding in small, isolated populations can translate into fitness reductions via inbreeding depression (Lacy 1987). Inbreeding depression can be expressed at different stages of a plant life cycle, and its strength depends on the mating system (Husband & Schemske 1996). Outbreeding plants are expected to be more susceptible to inbreeding depression than selfing plants, and especially to early inbreeding depression (Husband & Schemske 1996). For these reasons effects of recent habitat fragmentation on outbreeding species should become manifest already early in the life cycle, and they should be especially expressed in small and isolated populations of low genetic variability and high average relatedness among plants. Reduced genetic pollen diversity may also reduce progeny fitness via reduced pollen competition and sexual selection (Mulcahy 1979, Lee 1984, Marshall et al. 2000). In conclusion, increased inbreeding is one possible explanation for reduced plant fitness in small and isolated plant populations (Ellstrand & Elam 1993, Young et al. 1996).

Such an explanation requires that negative inbreeding effects are consistent among populations – or at least not stronger in larger and not isolated populations than in small and isolated ones. However, for several reasons inbreeding effects may differ among populations. In small and isolated populations of reduced genetic diversity, selfing may lead to higher homozygosity than selfing does in large populations, which may result in stronger negative fitness effects of selfing in small populations. Additionally, in small, isolated populations, purging could have reduced some of the genetic load, and the accumulation of mildly deleterious mutations could have increased negative effects (Lande 1995, Lynch et al. 1995). However, purging of genetic load and mutation accumulation are unlikely to play a role in the response to recent habitat fragmentation because of the long time spans required and because purging seems to be an inconsistent, if not ineffective mechanism (Frankham 1995, Lynch et al. 1995, Byers & Waller 1999). Although differential among-population effects of inbreeding in more and less fragmented habitats are of high ecological, evolutionary and conservation interest, they have rarely been studied, possibly because it involves experimentation in many field populations (Levin 1984, Paschke et al. 2002).

We studied effects of inbreeding and outbreeding on early stages in the life cycle for plants of the fen grassland specialist *Swertia perennis* in 16 calcareous fen grasslands in NE Switzerland (Caricion davallianae alliance; Ellenberg 1978). Calcareous fen grasslands are species-rich wetlands and belong to the few remaining semi-natural ecosystems of Central Europe (Pauli et al. 2002). They harbour nearly 50% of all endangered plant species of Switzerland (Landolt 1991). In Switzerland the overall wetland area was reduced by 90% since 1800 (Broggi & Schlegel 1989), and the remnants are highly fragmented (Hintermann 1992). Wetland populations are thus well suited for investigating habitat fragmentation effects on habitat specialists.

Today, *S. perennis* still grows abundantly in many fen grasslands. However, in a related study, we found that 24% of 63 populations of *S. perennis* had disappeared during the last 127 years (Lienert et al. 2002a). In sites with unchanged land use, local population extinction was attributed to habitat fragmentation. Isozyme analysis revealed relatively large overall genetic differentiation of the populations used for this study ($F_{ST} = 0.128$). Within-population inbreeding coefficients F_{IS} were highest and measures of genetic variability (mean number of alleles per polymorphic locus, mean observed heterozygosity) lowest in small and isolated populations (Lienert et al. 2002b). Furthermore, several components of fitness and density mea-

sures of *S. perennis* were significantly reduced in small and isolated populations (Lienert et al. 2002c).

We studied the early fitness measures seed production and germination after free pollination, hand-outcrossing and hand-selfing of *Swertia perennis* in large MAIN, in small barely isolated NEAR and in small isolated DISTANT habitat islands. As a control we also tested for apomictic seed set. We asked the specific questions: 1) is *S. perennis* capable of apomictic reproduction? 2) Are seed production and germination of *S. perennis* reduced for hand-selfed flowers, compared to flowers outcrossed with pollen of one pollen donor, or to freely pollinated flowers? 3) Do effects of pollination treatments differ between populations on small and isolated habitat islands, small and barely isolated habitat islands, and large habitat islands?

Materials and methods

Species

Swertia perennis L. (Gentianaceae) is a long-lived, iteroparous, herbaceous perennial that grows in calcareous fen grasslands and very wet grasslands (Hegi 1906). Adult rosettes have ovate, parallel-veined leaves and can develop daughter rosettes from lateral meristems. Reproductive shoots consist of a single, 15–60 cm tall stem with up to 30 light-purple, actinomorphic, star-shaped flowers in a thyrse, which flower in July and August. The protandrous flowers with eight to ten nectar chambers are visited by various insects (including species of the Coleoptera, Lepidoptera, Diptera (especially Syrphidae) and Hymenoptera (especially *Bombus* and Vespidae) J. Lienert, personal observation). Anthers open in the morning, when pollinating insects show large activity. Pollen is bright yellow in closed anthers and changes to reddish-brown when anthers open. The tips of the stigmas clearly separate when these are receptive (J. Lienert, personal observation), hence effective hand pollination is easy. Up to 50 winged seeds develop in one ovate fruit capsule (Hegi 1906). The growth period of *S. perennis* lasts from April–May, when aboveground plant parts emerge, until sites are covered by snow, typically in November.

Swertia perennis has a wide, but discontinuous distribution from Central Europe through Asia to western North America (Hulten & Fries 1986). In Switzerland, it only occurs in pre-alpine fen grasslands north of the Alps (Welten & Sutter 1982). It is considered endangered worldwide (Jäger & Hoffmann 1997), but can be locally abundant. Therefore, the Red List classification of *S. perennis* ranges from not endangered to highly endangered or even extinct among different Swiss regions (Landolt 1991).

Study sites

The hierarchical study design comprised 18 calcareous fen grasslands between 900 and 1340 m a.s.l. in the main distribution range of *S. perennis* in Switzerland. The study sites were equally divided between the two regions St. Gallen (SG) and Schwyz (SZ) and were randomly selected (apart from design restrictions; see Hooftman et al. 1999 for details of the study design used in a larger project on wetland fragmentation). In each region we studied three 'fen systems'. Each fen system consisted of three fen grasslands: one large MAIN habitat island (> 5 ha), which was associated with two smaller habitat islands (< 0.5 ha; Table 1). The small NEAR island was located at 40–125 m from its MAIN, whereas the small DISTANT island was separated by about 1000 m from its MAIN, and by more than 1000 m from any other fen grassland. Woodland or pasture vegetation separated the individual sites. Our study design followed the assumption that the dispersal of pollen or seeds for more than 1000–2000 m is very unlikely, even over the same habitat type (Levin 1988). Hence, the DISTANT islands are presumably strongly isolated, whereas the NEAR populations are subject to sporadic gene flow. Indeed, population differentiation between the study populations was confirmed in an earlier isozyme study (Lienert et al. 2002b). Population sizes of *S. perennis* on the MAIN islands ranged from 758 to 118500 flowering adults and from 7 to 6738 flowering adults on the NEAR and DISTANT islands (Table 1). One DISTANT island did not host a population of *S. perennis* (Ebnat-Kappel), and a second DISTANT island (Unteriberg) was mown before we could collect the capsules of our pollination experiment (see below). Therefore, our study comprised *S. perennis* in 16 fen grasslands.

Pollination experiments

We prepared bags for pollinator exclusion sewn from pollen-proof gauze fabric, which we could place around experimental flowers and carefully close with threads. In the experiment, we removed anthers with a pair of fine forceps, which we sterilised between treatments in a flame. For hand-pollination we gently brushed an open anther with ripe pollen over an open stigma until it was fully saturated with pollen.

1) In late July 1998 we tested for apomictic seed set by emasculating and bagging one flower on each of two different flowering plants per population when anthers and stigmas were still closed. 2) For the main experiment, we selected ten more flowering plants in each of the 16 populations (except for population Sattel DISTANT, which only had seven reproductive

adults; Table 1). As a control for the effect of free pollination, we marked one flower per plant with gardening wire. To test for effects of outcrossing with pollen of one pollen donor, we marked a second flower with an open stigma on the same plant with differently coloured gardening wire and pollinated it with ripe pollen of a plant at a distance of 5–10 m. This distance had turned out to be the optimal outcrossing distance in other plant species (Waser & Price 1989, Fischer & Matthies 1997). The high number of populations in the experiment did not leave us with sufficient time to also bag the outcrossed flowers. However, the large amount of pollen used for hand-outcrossing presumably saturated stigmas, so that additional pollen deposited by pollinators most likely did not contribute much to fertilisation (see Snow 1986, Groom 1998).

In contrast, the pollen deposited by insects presumably did not fully saturate stigmas (J. Lienert, personal observation). Hence, the pollen fertilising in the outcrossing treatment most likely represented one paternal plant, while pollen involved in free pollination most likely represented several paternal plants. To test for effects of selfing, we emasculated and bagged a third flower with closed anthers and stigma, which we pollinated with ripe pollen of a different flower of the same plant after one or two days, when the stigma was open.

In late August 1998, when seeds had matured, we collected the treated capsules. We dried them at room temperature for six weeks. We counted the number of undeveloped ovules and seeds per capsule and calculated the seed/ovule ratio. The number of ovules per

Table 1. Location (Swiss canton in parentheses), habitat island type (large MAIN, small, barely isolated NEAR and small, isolated DISTANT island), name of site, coordinates as in Swiss topographical maps, altitude, distance of small island (NEAR/DISTANT) to MAIN island, site size, areal extent of population and population size (number of flowering adults in 1998) for the 18 study sites of *Swertia perennis*. There were no *S. perennis* on the DISTANT island of Ebnat-Kappel, and the DISTANT island of Unteriberg was mown before capsules were collected

System	Island type	Fen grassland	Large coordinate	Small coordinate	Altitude (m a.s.l.)	Dist. to MAIN (m)	Island size (ha)	Areal extent (ha)	Popul. size
Grabs (SG)	MAIN	Hirzenbäder	747'650	227'300	1'340	–	5.73	3.00	118'500
	NEAR	Herti	747'550	227'025	1'340	45	0.20	0.11	917
	DISTANT	Maienberg	748'300	226'575	1'190	800	0.45	0.10	1'287
Wildhaus (SG)	MAIN	Bilchenmoos	748'100	230'400	1'210	–	6.51	6.51	65'110
	NEAR	Sommerigweid	748'300	230'525	1'230	40	0.57	0.16	2'772
	DISTANT	Schönenboden	746'950	230'300	1'130	925	0.52	0.20	1'562
Ebnat-Kappel, Hemberg (SG)	MAIN	Chellen, large	730'700	237'350	1'080	–	4.28	4.28	32'100
	NEAR	Chellen, small	730'425	237'175	1'070	106	0.54	0.33	6'738
	DISTANT	Allmeindswald	731'550	237'625	1'070	750	0.20	–	–
Einsiedeln/ Alpthal (SZ)	MAIN	Eigenrieter, large	697'850	215'900	990	–	7.47	0.60	2'400
	NEAR	Eigenrieter, small	697'750	215'750	990	44	0.20	0.09	985
	DISTANT	Etteren	696'975	213'250	1'020	1825	0.87	0.20	1'348
Unteriberg, Studen (SZ)	MAIN	Chilenried, large	705'600	214'750	895	–	5.43	0.51	758
	NEAR	Chilenried, small	705'500	214'675	900	125	0.66	0.08	250
	DISTANT	Ort	706'475	213'900	910	1200	0.18	0.18	4'446
Sattel (SZ)	MAIN	Zäll, large	692'050	213'975	1'130	–	6.68	2.66	21'256
	NEAR	Zäll, small	691'875	213'875	1'110	60	0.22	0.15	820
	DISTANT	Gigersberg	691'275	213'500	1'010	800	0.57	0.002	7

Table 2. Skeleton hierarchical statistical model of effects of pollination treatment and island types. Island type denotes a large MAIN (M), a small, barely isolated NEAR (N) and a small isolated DISTANT (D) island. This experimental set-up was replicated six times in the landscape. However, two DISTANT islands could not be used for the analysis, leaving 16 populations. Treatment denotes three different treatments on one individual, i.e. free pollination, hand-outcrossing and hand-selfing

Sources of variation	Factor levels	df	Variance ratios (F values)
Factor frugivory	Capsules with / without frugivory	1	$ms_{frugivory}/ms_{residual}$
Island type	3 habitat island types (M, N, D)	2	$ms_{island}/ms_{population}$
Population	16 populations	13	$ms_{population}/ms_{individual}$
Individual plant	Max. 10 individuals in each population	143	$ms_{individual}/ms_{residual}$
Treatment	3 treatments (free/outcrossing/selfing)	2	$ms_{treatment}/ms_{residual}$
Island – by – treatment interaction	Interaction	4	$ms_{island \times treatment}/ms_{population \times treatment}$
Population – by – treatment interaction	Interaction	26	$ms_{population \times treatment}/ms_{residual}$
Residual		286	

Note: df = degrees of freedom, ms = mean square

capsule (number of seeds per capsule + number of undeveloped ovules per capsule) did not differ among treatments (ANOVA, $F_{2,169} = 1.140$, $P > 0.3$). Then we weighed the seeds of each capsule to the nearest µg and obtained individual seed mass by dividing the total seed mass per capsule by the number of seeds per capsule. Moreover, we estimated the degree of damage by frugivores of individual capsules by classifying them into the three categories no frugivory (category 1; 297 capsules), some traces of frugivory (i.e. hole, threads or insect in capsule; (category 2; 54 capsules) and entire capsule consumed, no seeds left (category 3; 94 capsules).

In October 1998, we placed the seeds on wet Vermiculit substrate (Vermex, Vermica AG, 5076 Bözen, Switzerland) in petri dishes. We kept them in the dark at +4 °C for five days, stratified them at –4 °C for four weeks, let them thaw at +4 °C for another four days and then let them germinate for six weeks in a climate chamber with 14 hours of light and 10 hours of darkness. The temperature was 20 °C during daytime and 10 °C in the dark, except for week four to six, where it was 23° and 18 °C, respectively. We randomised the petri dishes before the beginning of germination and after two weeks. After ten days seedlings began to germinate and after five weeks, hardly any additional seedlings appeared. Hence, we calculated germination percentage as the percentage of seeds that had germinated after six weeks. Although few seeds may have been dormant we are confident that we were able to break seed dormancy with our stratification procedure, because only seeds that did not look viable did not germinate. Therefore, we regard germination percentage as indicator of seed viability.

Data analysis

We analysed all data with hierarchical analyses of variance (ANOVA) or logistic regression using the computer program GenStat 5 (release 4.2; Payne et al. 1993). In case of logistic regression, we treated mean deviance changes like mean squares in an ANOVA, since deviance-change ratios approximately follow the F distribution (Payne et al. 1993). For logistic regression we used the complementary-log-log link as described in Egli & Schmid (2001).

In the main experiment, we tested for effects of the three island types, the 16 populations, the 160 maternal plants (10 per population) and the three treatments (see skeleton model; Table 2). Additionally, we used contrasts to test, whether effects of island type (MAIN, NEAR, DISTANT) were rather due to the factors habitat isolation or to small habitat area. To this end, we split the two degrees of freedom for the three island types in both possible ways: in one test, we fit-

ted habitat isolation after correcting for habitat area. In the other test, we fitted habitat area after correcting for habitat isolation. This means that we used the two degrees of freedoms in two tests as following: (a) test for isolation; contrast 1 = MAIN vs. (NEAR + DISTANT); contrast 2 = NEAR vs. DISTANT. (b) Test for area; contrast 1 = (MAIN + NEAR) vs. DISTANT; contrast 2 = MAIN vs. NEAR. Analogously, we analysed contrasts to explore effects of selfing versus outcrossing, of outcrossing versus free pollination, and of free pollination versus selfing. To test, whether the performance after outcrossing differed between island types, we also analysed island effect and contrasts for outcrossed fruits separately.

Because there is often a trade-off between the number of seeds per capsule and seed mass, we tested for such a relationship, and included the number of seeds as covariate in the analysis of seed mass.

The degree of frugivory of capsules was higher on outcrossed than on selfed (+39%) and freely pollinated flowers (+28%; both $P < 0.001$). The difference between selfed and outcrossed flowers may have arisen from bagging only the selfed flowers. Frugivory was 23% lower on DISTANT than on MAIN and NEAR islands (contrast for isolation: $P < 0.01$). It is thus conceivable, that frugivory interfered with treatment effects on seed production and germination, although data on seed production and germination were of course only available for capsules with no or little frugivory, i.e. for those 351 (out of 445) capsules of categories 1 and 2. Nevertheless, to correct for potential interference of frugivory with our treatments, we also analysed data on seed production and germination with the degree of frugivory as a factor. The low number of deviating results between analyses with and without factor frugivory indicates that frugivory does not account for the treatment effects reported below. In the text, we present the results of analyses without including frugivory and mention the few deviating results from analyses including this factor.

Results

Test for apomixis

None of the 32 flowers in the 16 populations that was emasculated and bagged developed a seed. Unless *S. perennis* were pseudogamous this indicates that it is not apomictic.

Effects of habitat island types

The measures of seed production and germination percentage did not differ between island types (Table 3A).

The same held true, when only outcrossed plants were analysed.

Effects of pollination treatments

Pollination treatments significantly affected all variates (Tables 3A, 4). Compared to hand-outcrossing, hand-selfing reduced the number of seeds per capsule by 17% (P < 0.05, and P < 0.01 with factor frugivory; Tables 3B, 4, Fig. 1A), reduced the seed/ovule ratio by 23% (Fig. 1B) and seed mass by 20% (both P < 0.001; Fig. 1C), but did not significantly affect germination percentage, which was reduced by 3% (Fig. 1D).

Compared with free pollination hand-outcrossing with one pollen donor reduced seed mass by 7% (P < 0.01, and with factor frugivory, P < 0.05; Tables 3B, 4, Fig. 1C) and germination percentage by 21% (P < 0.05 without covariate, and not significant with the factor frugivory; Fig. 1D).

Finally, the differences in the outcomes of free pollination and hand-selfing were the most marked ones:

hand-selfing reduced the number of seeds per capsule by 22% (P < 0.01 and P < 0.001 with factor frugivory; Tables 3B, 4, Fig. 1A), the seed/ovule ratio by 26% (Fig. 1B), seed mass by 25% (both P < 0.001; Fig. 1C), and germination percentage by 23% (P < 0.05 and P < 0.01 with factor frugivory; Fig. 1D).

In summary, we found a distinct gradient from freely pollinated, over hand-outcrossed with one pollen donor, to hand-selfed flowers (Table 4, Fig. 1). This gradient always pointed in the same direction: the higher the degree of inbreeding, the higher was the observed fitness reduction.

Pollination treatment effects in populations of different habitat island types

Compared with hand-outcrossing, the reduction in the number of seeds per capsule due to selfing appeared most pronounced on DISTANT islands (P < 0.1, with factor frugivory P < 0.05). Moreover, compared with hand-outcrossing the reduction in seed mass due to

Table 3. Effects of pollination treatment on reproduction, germination and frugivory in *Swertia perennis*. Results of logistic regression and hierarchical ANOVA of frugivory (in three categories; 1: no frugivory of capsule; 2: some traces of frugivory (i.e. hole, threads or insect in capsule); and 3: entire capsule subject to frugivory, no seeds left), number of seeds per capsule, seed/ovule ratio, seed mass and percentage of seeds germinated after six weeks. A: test for differences among the three habitat island types (i.e. large MAIN, small, barely isolated NEAR and small, isolated DISTANT island), the 16 populations, the individual plants (maximally 10 in each population), the treatments (free pollination, hand-outcrossing and hand-selfing) and for interactions. The degrees of freedom (df) for individuals and residuals, respectively, were: 137/260 (frugivory), 128/169 (no. of seeds/capsule, seed/ovule ratio), 120/156 (seed mass), 120/154 (germination percentage). B: contrasts to test for effects between selfing and outcrossing, outcrossing and free pollination and free pollination and selfing (see methods). We present mean squares (ms) or mean deviance changes (mdev), and P values: + P < 0.1; * P < 0.05; ** P < 0.01; *** P < 0.001

A	Sources of variation	Test	df	Frugivory (3 levels) mdev	No. of seeds/ capsule ms	Seed/ovule ratio mdev	Seed mass ms	Germination percentage mdev
	Island	A	2	12.92	76.06	0.251	0.053	0.653
	Population		13	8.17 ***	399.73 *	0.712	0.066 ***	2.521 ***
	Individual plant		144	1.90 ***	191.53 ***	0.619 ***	0.011 ***	0.491
	Treatment	B	2	21.13 ***	334.20 *	4.693 ***	0.164 ***	1.270 *
	Island - by - treatment interaction	A × B	4	1.33	127.31	0.035	0.009	0.297
	Population - by - treatment interaction		26	1.76 **	185.80 ***	0.430 *	0.011 **	0.854 **
	Residual		288	0.82	70.78	0.258	0.005	0.413

B	Treatment contrasts		df	Frugivory (3 levels) mdev	No. of seeds/ capsule ms	Seed/ovule ratio mdev	Seed mass ms	Germination percentage mdev
	Selfing vs. outcrossing		1	41.16 ***	283.6 *	5.196 ***	0.099 ***	0.013
	Outcrossing vs. free		1	14.86 ***	34.4	0.070	0.044 **	1.863 *
	Free vs. selfing		1	5.83 **	634.7 **	8.074 ***	0.327 ***	1.697 *

Table 4. Mean effects of pollination treatment on frugivory, seed production and germination in *Swertia perennis*. The observed traits were frugivory (in three categories; 1: no frugivory of capsule; 2: some traces of frugivory (i.e. hole, threads or insect in capsule); and 3: entire capsule subject to frugivory, no seeds left), number of seeds per capsule, seed/ovule ratio, seed mass (mg) and percentage of seeds germinated after six weeks. We show means with standard errors in parentheses (± se). The levels of significance of treatment differences are presented in Table 3B

	Frugivory (3 levels) mean	± se	No. of seeds/capsule mean	± se	Seed/ovule ratio mean	± se	Seed mass mean	± se	Germination percentage mean	± se
Free	1.458	(0.065)	21.77	(0.97)	0.780	(0.020)	0.301	(0.010)	40.83	(3.08)
Outcrossing	1.864	(0.074)	20.40	(1.23)	0.743	(0.024)	0.280	(0.012)	32.24	(3.45)
Selfing	1.336	(0.056)	17.01	(1.08)	0.575	(0.030)	0.225	(0.009)	31.40	(2.92)

selfing appeared most pronounced on DISTANT islands and lowest on NEAR islands (both P < 0.05). However, the overall island – by – treatment interaction was not significant for any measure (Table 3A), suggesting that effects of hand-pollination and of selfing were independent of habitat size and isolation.

The number of seeds per capsule was negatively correlated with seed mass (Pearson's correlation, r = –0.173, N = 280, P < 0.01), indicating a trade-off between the two. However, the outcome of the analysis of seed mass with the covariate 'number of seeds per capsule' did not differ in levels of significance from the one without the covariate; which is presented in Table 3A.

Significant variation among the 16 populations and among the individual plants, and the significant population – by – treatment interaction (see Table 3A), indicate that variation other than the one caused by habitat fragmentation, i.e. other than between island types, also influenced treatment effects on seed production and germination of *S. perennis*.

Discussion

Effects of pollination treatments

Higher seed production after free pollination than after experimental outcrossing with ample pollen suggests that natural reproduction in *S. perennis* is not pollen limited. This is in line with its generalised pollination system. Since it offers plenty of nectar as reward to many different pollinator taxa, it attracts many pollinating insects to its wide-open flowers. Because insects
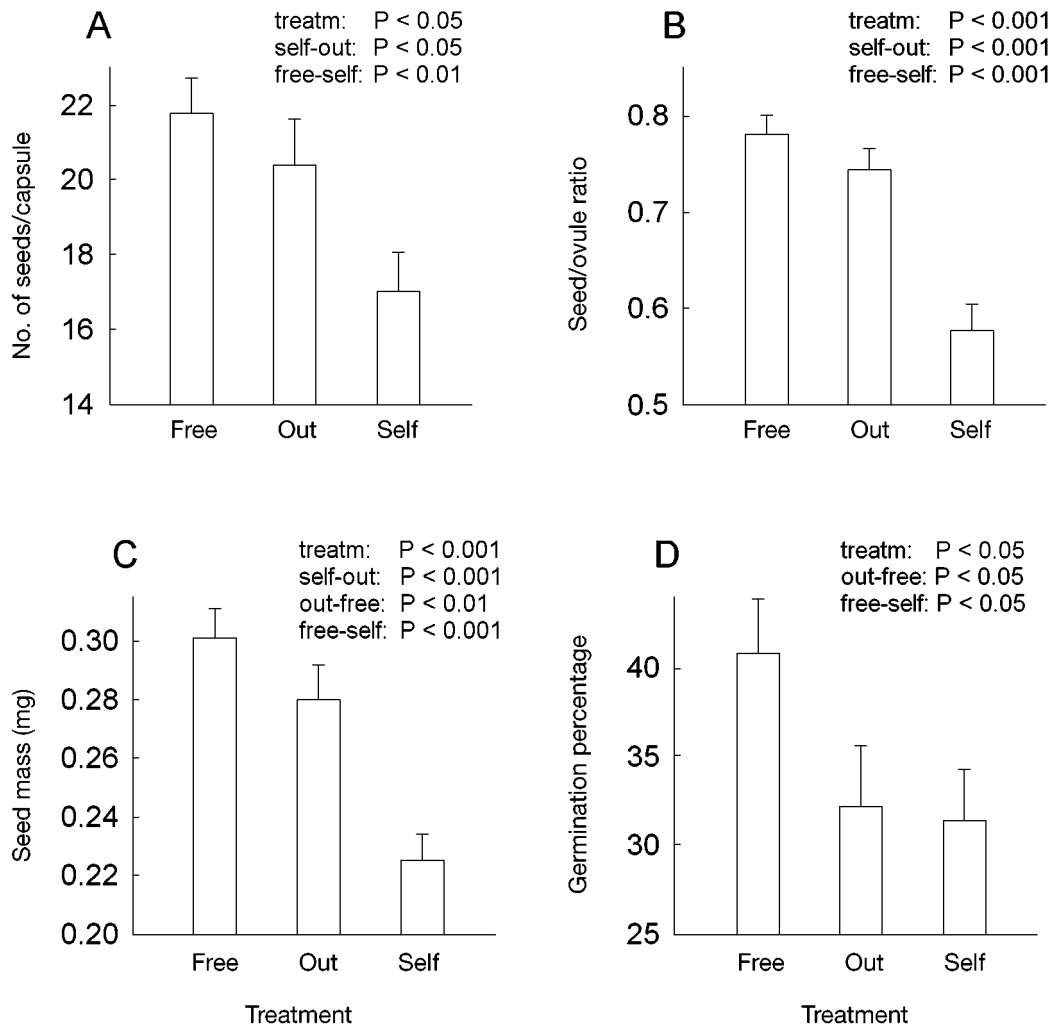

Figure 1. Effects of pollination treatments on seed production and germination using plants in 16 populations of *Swertia perennis*. The treatments were free pollination, hand-outcrossing and hand-selfing. We indicate significance levels for overall treatment effects (treatm), for the effect of selfing vs. outcrossing (self-out), of outcrossing vs. free pollination (out-free) and of free pollination vs. selfing (free-self). A: number of seeds per capsule, B: seed/ovule ratio, C: seed mass, D: percentage of seedlings germinated after six weeks. Error bars denote 1 se; note: the y-axes do not start at the origin.

are abundant even in the small and isolated study populations and frequently visit several plants of *S. perennis* (J. Lienert, personal observation), natural pollination of *S. perennis* may be accomplished through several pollen donors. Thus, *Swertia perennis* may be less sensitive to fragmentation-caused loss of specialist pollinators or to reduced pollinator diversity than more specialised plants (Bond 1995, Waser et al. 1996).

We found a distinct gradient of reduced seed production and germination percentage from free pollination over hand-outbreeding with one pollen donor to hand-selfing for seed number, the seed/ovule ratio, seed mass and germination percentage (Tables 3, 4; Fig. 1). Since only the selfed flowers were bagged, a confounding between selfing treatment and bagging is possible, e.g. by changing microclimatic conditions or shading developing fruits. Therefore, lower reproductive success after selfing may partially be an artefact and should be verified in controlled experiments. In contrast, we did not bag the flowers of both outcrossing treatments. Moreover, pollen limitation cannot explain the outcome of our experiment, since treated stigmas were always saturated with pollen and because it would not explain effects on germination percentage. We conclude that increased levels of inbreeding increasingly reduced fitness, or in other words, that reduced pollen quality from free pollination over hand-outcrossing to hand-selfing was largely responsible for the observed treatment effects.

Our results follow the expectation that increased among-plant relatedness in outcrossing plant species reduces early offspring fitness because of inbreeding depression (Husband & Schemske 1996). Moreover, seed provisioning might have been reduced after inbreeding, which may change maternal effects that can strongly affect early stages in the life cycle (e.g. Schaal 1984, Roach & Wulff 1987, Waser et al. 1995). Furthermore, reduced pollen competition and sexual selection after pollination with less diverse pollen (Mulcahy 1979, Lee 1984, Niesenbaum 1999, Pflugshaupt et al. 2002) may have contributed to reduced fitness after inbreeding. To assess the relative importance of the different potential mechanisms affecting seed production and germination more sophisticated diallel or reciprocal crosses or multigeneration designs are required (Shaw & Waser 1994).

Further experiments would also help to explain the higher reproductive success of free pollination compared with the outcrossing treatment. Two potential mechanisms may be responsible. First, several pollen donors may be involved in free pollination leading to high diversity of pollen, whereas we used only one pollen donor in our treatment. This could be indicative that *S. perennis* is largely outcrossing in natural populations. Higher pollen diversity involved in free pollination may be especially beneficial for offspring fitness, because it allows for pollen competition and sexual selection, which increase the likelihood of fertilisation by favourable pollen (Mulcahy 1979, Lee 1984, Marshall et al. 2000). Alternatively, in free pollination the pollen could have been of higher quality because the transferred pollen stemmed from plants less related to the target plants than the donors used in our hand-outcrossing treatment. The latter grew in 5–10 m distance from our target plants. Inbreeding depression after experimental short-distance crosses was observed in several plant species (e.g. Waser & Price 1989, Dudash 1990, Fischer & Matthies 1997, Paschke et al. 2002) and indicates that populations are not panmictic, but have a neighbourhood structure (e.g. Schaal 1980, Crawford 1984, Levin 1984).

Effects of size and isolation of habitats

Populations responded differently to pollination treatments for number of seeds per capsule, seed mass and germination percentage, but this was independent of their island type (Table 3A). Hence, there was evidence neither of increased inbreeding depression in small and isolated populations of *S. perennis* nor of purging of detrimental alleles from such populations.

In some plant species consistent strength of inbreeding depression in populations of different size, combined with increased levels of inbreeding in smaller populations may explain the reduced reproductive success observed with decreasing population size (Menges 1991, Widén 1993, Fischer & Matthies 1998, Oostermeijer et al. 1998, Kéry et al. 2000, Mavraganis & Eckert 2001). However, in other studies no such relation was found (Ouborg & van Treuren 1994, van Treuren et al. 1994, Hauser & Loeschcke 1994, Kahmen & Poschlod 2000). In a related demographic study on *S. perennis*, plant density and vegetative traits such as number and size of leaves and stem height were reduced in DISTANT islands (Lienert et al. 2002c). Because within-population inbreeding coefficients (F_{IS}) were highest and genetic variability significantly lowest in DISTANT populations (Lienert et al. 2002b) this may indicate that inbreeding depression plays an even more important role at later life stages than at the very early ones studied here. Alternatively, the reduced vegetative growth of *S. perennis* in DISTANT islands may have had a non-genetic reason. Reduced seedling establishment (Leiss & Müller-Schärer 2001) in DISTANT islands would be consistent on the one hand with reduced plant density but largely unaffected adult plant size reported earlier (Lienert et al. 2002c), and on the other with negative effects of higher relatedness among crossing plants in such islands as suggested by this study.

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