



Effects of inbreeding and pollen donor provenance and diversity on offspring performance under environmental stress in the rare plant *Cochlearia bavarica*

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Summary

Habitat degradation and loss can reduce size and genetic variability of natural populations, increasing individual homozygosity and average relatedness between individuals. While the resulting inbreeding depression may be reduced by natural selection under prevailing environmental conditions, it may increase again under environmental stress. To investigate the effect of environmental stress on offspring performance and the expression of inbreeding depression, we hand-pollinated maternal plants in small (< 100 , $n = 5$) and large populations (> 400 flowering plants, $n = 5$) of the rare plant *Cochlearia bavarica* (Brassicaceae) and raised the offspring under experimentally manipulated water and light regimes (normal or reduced supply). In addition to considering natural variation in inbreeding levels due to population size, we manipulated pollen donor provenance and diversity. Maternal plants were pollinated with nine donors from a different population or with one or nine donors from the same population. One further inflorescence of each maternal plant was exposed to free pollination. Offspring growth and survival were monitored over 300 days. Offspring performance varied significantly among populations and maternal plants. Environmental stress interacted significantly with these factors. However, there was no general indication that offspring from small populations were more negatively affected. In seven out of 10 populations, offspring derived from between-population pollination performed better than offspring derived from within-population pollination. Also, in five out of 10 populations, average offspring size was

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higher after within-population pollination with nine than after pollination with one pollen donor. These results suggest low genetic diversity within *C. bavarica* populations, both smaller and larger ones. Interactions between environmental stress and pollination treatment indicated that using pollen donors from outside a population or increasing the number of pollen donors can reduce inbreeding depression, but that this beneficial effect is impaired under stressful conditions.

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Zusammenfassung

In kleinen Pflanzenpopulationen kann genetische Erosion zu einer zunehmenden Ähnlichkeit zwischen den Pflanzen innerhalb dieser Populationen führen. Die daraus resultierende Inzuchtdepression kann unter den bestehenden Umweltbedingungen durch die natürliche Selektion reduziert werden, kann aber unter Umweltstress erneut ansteigen. Um diese Hypothese zu testen, wurden Pflanzen kleiner (< 50 blühende Pflanzen, $n = 5$ Populationen) und großer Populationen (> 400, $n = 5$) der endemischen Art *Cochlearia bavarica* handbestäubt. Die aus den Handbestäubungen resultierenden Keimlinge wurden unter verschiedenen Umweltbedingungen aufgezogen (experimentelle Manipulation von Licht und Wasser: normale und reduzierte Bedingungen). Um zusätzlich zur Variation der Populationsgröße auch unterschiedliche Grade von Inzuchtdepression zu erzeugen, wurden die Mutterpflanzen mit Pollen von neun Pollenspendern aus einer anderen Population oder mit einem oder neun Pollenspendern aus der gleichen Population handbestäubt. Das Wachstum der Nachkommen wurde über 300 Tage beobachtet. Die Größe der Nachkommen variierte signifikant zwischen den verschiedenen Ursprungspopulationen und Mutterpflanzen. Zusätzlich reagierten die Ursprungspopulationen (und Mutterpflanzen) unterschiedlich auf den experimentell erzeugten Umweltstress, aber nicht im erwarteten Sinne einer Benachteiligung kleiner gegenüber großer Populationen. Nachkommen, die aus Handbestäubungen mit neun Pollenspendern einer benachbarten Population stammten, waren in sieben von zehn Populationen signifikant größer als die Nachkommen, die aus Handbestäubungen innerhalb der Populationen hervorgingen. Nachkommen, die aus Handbestäubungen mit einem Pollenspender aus derselben Population hervorgingen, waren in fünf von zehn Populationen kleiner als die Nachkommen aus einer Handbestäubung mit neun Pollenspendern aus derselben Population. Dies weist darauf hin, daß die genetische Vielfalt innerhalb aller *Cochlearia bavarica*-Populationen relativ niedrig war, unabhängig von deren Größe. Pollen von außerhalb einer Population oder eine zunehmende Anzahl an Pollenspendern können Inzucht und Inzuchtdepression vermindern. Signifikante Interaktionen zwischen Umweltstress und Handbestäubungsbehandlung zeigten aber auch, daß dieser positive Effekt unter Umweltstress beeinträchtigt werden kann.

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Introduction

Many plant and animal species occur in small remnant populations due to habitat loss and degradation (Vitousek, 1994). Because population size can be negatively correlated with genetic variation (Fischer & Matthies, 1998; Paschke, Abs, & Schmid, 2002a), a decrease in population size may result in genetic erosion (Oostermeijer, Berholz, & Poschlod, 1996). The underlying mechanisms of genetic erosion (loss of alleles through genetic bottleneck, inbreeding, or high rates of random genetic drift; Falconer, 1989), although conceptually distinct, are often correlated (Gabriel & Bürger, 1994).

Negative effects of inbreeding on individual performance have been documented for instance for reproductive traits and offspring establishment (Fishman, 2001; Lienert, Diemer, & Schmid, 2002; Pflugshaupt, Kollmann, Fischer, & Roy, 2002; Paschke, Abs, & Schmid, 2002b; Lienert & Fischer, 2004) and in various groups of organisms and environments (Armbruster, Hutchinson, & Linnell, 2000; Andersson & Waldmann, 2002; Haag, Hottinger, Riek, & Ebert 2002; Keller & Waller, 2002). These effects may be due to recessive alleles that are deleterious when homozygous (partial dominance hypothesis), an advantage in fitness of heterozygotes compared to homozygotes (overdominance hypothesis; Charlesworth

& Charlesworth, 1987), or an increase in developmental instability in homozygotes (but see Clark, 1993). After inbreeding, selection against deleterious alleles may purge populations from their genetic load (Husband & Schemske, 1996), especially in small populations. In small populations drift may fix mildly deleterious mutations even against selective sweeps (Charlesworth, Nordborg, & Charlesworth, 1997). Selection will purge lethal or sublethal mutations, while mutations of small or additive effects are under weak selective pressure and will accumulate (reviewed in Byers & Waller, 1999; examples: Paland & Schmid, 2003).

Although inbreeding depression was long considered to be independent of the environment, there is increasing awareness that inbreeding depression may be stronger under harsh than under benign environmental conditions (Bijlsma, Budgaard, & Van der Putten, 1999). Populations living in harsh environments should experience different selective pressures and express more pronounced inbreeding depression than populations living in conditions closer to the species optimum (Levin, 1984). Even if populations are exposed to purging, this process might only be effective under specific environmental conditions, and environmental change may still result in the expression of inbreeding depression (Bijlsma et al., 1999).

To investigate the effect of environmental variation on individual performance and the expression of inbreeding depression in the rare *Cochlearia bavarica*, we conducted hand-pollinations in the field and exposed the offspring to four experimental environments in the greenhouse. This was repeated for 10 populations of small or large size because natural inbreeding levels might decrease with increasing population size. Known populations of these rare endemite (see Study species) range in size from 10 to more than 2000 flowering plants and, especially populations with few flowering individuals, are characterized by low genetic diversity (Paschke et al., 2002a). In a population with low genetic diversity, it may be advantageous for a maternal plant to receive pollen from several donors, some of which will be less related to it than the average (Paschke et al., 2002b; Bernasconi, 2004; Bernasconi et al., 2004). When pollen of different donors compete on the stigma for fertilization, the less closely related pollen may be at a competitive advantage, resulting in fewer inbred offspring and reduced inbreeding depression (Souto, Aizen, & Premoli, 2002). However, only genetic analysis of paternity, which has rarely been done in plants, would ultimately reveal whether post-pollination selection favors less related donors

(Bernasconi, Paschke, & Schmid, 2003; Bernasconi, 2004). Additionally, if mating within a population is accompanied by inbreeding, crossing with pollen from outside the population may further reduce inbreeding and its negative effects on offspring fitness. We attempted to experimentally reduce inbreeding levels in both small and large populations by using multiple pollen donors and pollen donors from outside the population of a maternal plant (Paschke et al., 2002b). We then compared the performance of the ensuing offspring varying in natural levels of inbreeding and diversity and provenance of pollen donors across different experimental environments.

Material and methods

Study species

Cochlearia bavarica (Brassicaceae, Vogt, 1985) is a herbaceous perennial plant that presumably arose from hybridization (*C. pyrenaica* x *C. officinalis*; Koch, Hurka, & Huthmann, 1996). It is a habitat specialist of calcareous springs with continuous cold water supply (Abs, 1999), and occurs in open calcareous fens, woodland clearings and shaded woodland springs. The species is endemic to Bavaria (Germany) with narrow distribution in two regions near Munich. At present, about 30 populations are known from 21 sites. One plant produces on average around 300 flowers (Paschke et al., 2002a).

Hand-pollination with variable pollen diversities and with pollen from foreign populations

In May 1998, we surveyed 22 populations and estimated population size as the number of flowering plants. For these populations, allozyme variation had been assessed in 1996 (Paschke et al., 2002a). We selected 10 populations: five large (> 400 flowering plants), and five small populations (< 50 flowering plants, Table 1). In each population we pollinated five randomly selected flowering plants by hand. On the primary inflorescence of each marked plant, we randomly selected four branches and bagged three of them with nylon fabric (mesh size <0.25 mm, Fig. 1). Three bagged branches were randomly assigned to hand pollination with (1) one pollen donor from the same population (low-diversity, within-population pollination), (2) a pollen mixture of nine donors from the same population (high-diversity,

within-population pollination), or (3) a pollen mixture of nine donors from a different population 100–1000 m away (high-diversity, between-population pollination). The unbagged inflorescence

Table 1. Study populations and environmental conditions at natural sites. Population size was estimated as the number of flowering plants (Paschke, 2002a) in repeated censuses (1996, 1998 and partly in 2000). Habitat types: I: woodland springs with tuffaceous limestone or gravel; II: woodland springs, fine soil, rich in organic material; III: calcareous fens; IV: river banks and ditches (see Abs, 1999)

Population	Location	Population size	Habitat type
A	Bäuerle	48	I
B	Gfällmühle	20	II
C	Ollarzried a	11	II
D	Ollarzried b	15	IV
E	Schönlings a	28	III
F	Kleinkemnat	1360	I
G	Ollarzried c	432	I
H	Schönlings b	2627	III
I	Katzbrui a	780	II
J	Katzbrui b	2057	I

branch was used as a free-pollination control. We marked pollen-donor plants in each population at the start of the experiment and used them for all successive hand pollinations. For low-diversity pollinations we randomly selected one pollen-donor plant in each population (≥ 0.5 m from the receiving plants). For the high-diversity, within-population pollinations we used sets of nine donors excluding the plants used for low-diversity pollinations. We also marked nine randomly chosen pollen-donor plants in populations for the high-diversity, between-population pollinations. Between-population pollen loads only consisted of pollen from one randomly assigned population other than that of the pollen-receiving plants. To ensure that we used the same quantity of pollen for each treatment, we emasculated 60–90 flowers (low-diversity pollinations) or 10 flowers per donor plant (high-diversity pollinations) and collected all the anthers in tubes. We dried the samples until the pollen grains were released from the anthers (≥ 1 h). We hand-pollinated all open flowers at each marked inflorescence with the corresponding pollen treatment, moving a soft brush with pollen over the stigma. We repeated the pollinations of the same plant with newly collected pollen in weekly intervals.

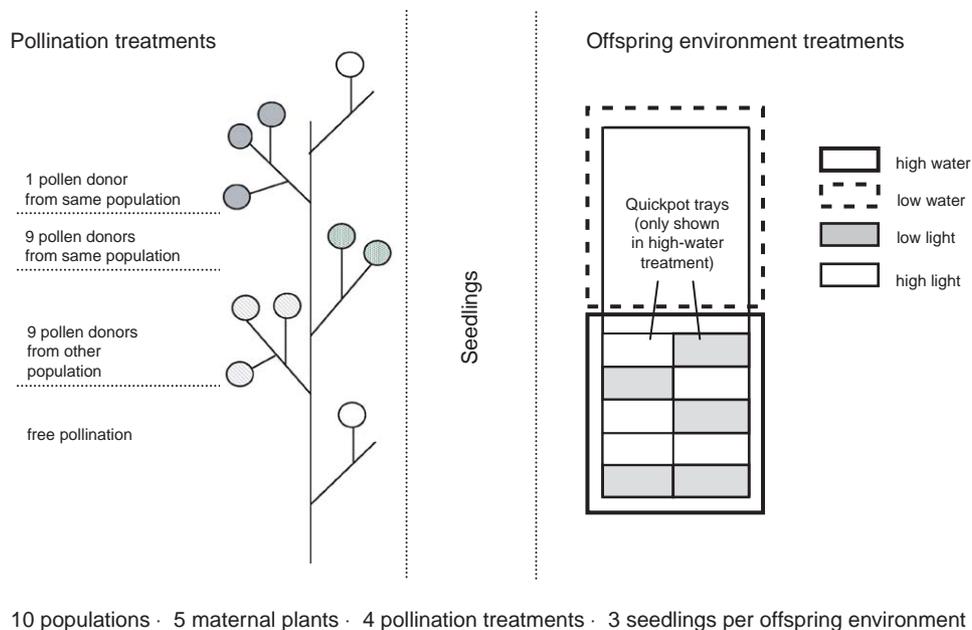


Figure 1. Temporal design of the greenhouse experiment on *Cochlearia bavarica*. The pollination treatments were randomly assigned to branches within inflorescences, a particular random assignment is shown in the sketch on the left (inflorescence branches had actually many more flowers than shown in the sketch, see “Material and methods”). A total of 117 seedling families resulting from the pollinations in five large (> 400 flowering plants) and five small populations (< 50 flowering plants) were grown on two tables in a greenhouse shown in the sketch on the right. Light (applied to trays) and water (applied to half-tables) treatments were factorially crossed to give the following offspring environments: high-water/high-light, high-water/low-light, low-water/high-light, low-water/low-light. From each combination three seedlings of each family (classified by maternal plant and pollination treatment) were randomly assigned to trays.

We estimated maternal plant size as the number of inflorescences per plant (Paschke et al., 2002a). Response variables of pollination treatments (fruit set, seed set, germination rate and offspring survival and performance in a common garden) were measured and analyzed in Paschke et al. (2002b). For the present study, addressing the effect of environmental stress on offspring performance, we collected 5–20 fruits per pollination treatment from each pollen-receiving plant in June 1998. We obtained 183 out of 200 potential seed families, since some pollinations did not result in seeds (see Results). All seeds (mean \pm SD = 31 ± 35 seeds/family) were dried and germinated on wet filter paper in a petri dish (light: 16 °C/14 h; dark: 10 °C/10 h). Thirty days after the start of germination tests, we measured the initial size (length of the longest leaf, nearest mm) of three randomly chosen seedlings within each petri dish. After another 21 days, the seedlings were individually planted in quickpot trays (54 plants per tray), transferred to the greenhouse and exposed to experimental conditions (see below). Day 1 of the experiment refers to the day on which plants were transferred to the greenhouse.

Greenhouse experiment with different offspring environments

Seedlings were exposed to a 2×2 factorial design, in which water supply and light intensity were manipulated to vary environmental stress (Fig. 1). Because *C. bavarica* is restricted to spring sites, we expected water supply to be most relevant for plant performance. In the field, light conditions also vary and this correlates with plant reproductive traits (Paschke et al., 2002a). In the high-water group (a priori the “normal environment”), we watered the plants 5 min/day, and the soil was always wet. In the low-water group (“stressful environment”), we watered the plants every 7 days for 10 min, so that the soil dried out between watering days. Within each water treatment, plants were either under full natural daylight (high light = “normal environment”), or light intensity was reduced by 53% (low light = “stressful environment”) using plastic boxes (WAVIBOX, 31620A, GVZ-BOLLTEC/CH). Natural light availability varied seasonally during the experiment.

Where possible, we planted three seedlings (in a few families only one or two) from each pollination treatment and seed family (in total $n = 1386$ seedlings from 117 seed families) into each offspring environment. We planted the seedlings in a completely randomized physical order to quickpot

trays ($n = 10$ or 11 trays with 54 pots per tray for each environment; 2:1-soil:sand; M. De Baat BV BF4 AB Coevorden/NL). On day 210 all plants were transplanted to larger-sized quickpot trays (35 pots per tray). The trays were arranged over two tables, and re-randomized between the tables at 4-week intervals (within the same environment) and between the trays when the plants were transferred to a new pot. On both tables we used half of the table for the high- and half for the low-water group (Fig. 1). The high- and low-light treatments were applied at tray level. Random arrangement of trays to each environment and re-randomization of tray position over time should minimize confounding between treatment and table effects.

We counted leaves and measured (nearest mm) plant height as the height of the longest leaf tip (see Fig. 1B in Paschke, Bernasconi, & Schmid, 2003) on day 60, 120, 210 and 270. As an overall estimate of plant performance we used total plant size defined as the product of the number of leaves and plant height. On day 120 and day 270, we also measured maximum length and maximum width of one randomly selected leaf per plant. Leaf area was defined as the product of leaf length and maximal width. We also recorded plant survival. Plant mortality was very low during the first 270 days. On day 270, the water supply in the greenhouse was interrupted for a week. Afterwards mortality was so high that on day 300 we harvested all plants and determined aboveground dry mass (after 65 °C for 120 h).

Statistical analysis

We analyzed the effects of population size (small vs. large populations), population identity (random effect, nested within population size group), pollination treatment, and offspring environment on total plant size and leaf area with repeated-measures analysis of variance (ANOVA) using a split-plot approach with averaged *F*-tests for within-subject effects (Hand & Taylor, 1987; as implemented in SPSS 6.1.1, 1995). Degrees of freedom were adjusted with Greenhouse–Geisser Epsilon (SPSS 6.1.1, 1995). In addition, offspring traits (i.e. final survival rate and dry mass measured once and each repeated measure of plant size and leaf area) were analyzed in univariate ANOVAs to get detailed insight into treatment effects at each measurement time. Terms were fitted sequentially and the obtained mean squares used to calculate appropriate ratios for *F*-tests. First, maternal plant size, germination rate, and mean initial seedling size (the latter two are means for each maternal plant),

and population heterozygosity (as estimated in Paschke et al., 2002a) were entered as covariates in the initial model of growth response of seedlings across experimental environments. The distributions of residuals of maternal plant size and germination rate deviated significantly from normality. These traits were thus square-root- and arcsine-square-root-transformed, respectively. Population size group was included after the tray effect and tested against population identity. Interactions of population size with environment and pollination treatment were fitted against the corresponding interaction terms with population identity. Offspring environment was tested against the random interaction environment \times population identity in repeated-measures ANOVA, but against the random effect of tray in the univariate ANOVAs. We also fitted the linear effect of offspring environment quality as measured by the mean performance of all plants in each environment at the particular time of measurement (stability analysis, Bell, Lechowicz, & Waterway, 2000), the deviation from this linear contrast, and all corresponding interactions. Mean performance per environment was different for each measurement period and therefore this linear effect was not considered for repeated-measures ANOVA.

In univariate ANOVAs, pollination treatments were decomposed into three orthogonal contrasts (Table 2). The first contrast distinguished free pollination from hand pollination (= C1), to control for the effect of hand-pollination itself. The second contrast compared low versus high pollen diversity within the hand-pollination treatments (= C2), and thus tests whether increasing pollen diversity can reduce inbreeding depression. The third contrast compared within- versus between-population pollinations of high diversity (= C3), to test whether using pollen from a different

population can reduce inbreeding depression. Contrast effects and their interactions with environment and population size were tested against the corresponding interaction terms with population identity. Population size itself was tested against maternal plant identity.

The explanatory terms in the ANOVAs were considered to reveal the following sources of observed phenotypic variation (Pigliucci, Schlichting, & Whitton, 1995). Environmental effects point to phenotypic plasticity (adaptive or non-adaptive). Population size group, population identity, and maternal plant identity effects indicate genetic variation in phenotypic traits. Interactions between environment and population size, population identity, or maternal plant identity indicate that responses to different environments ("reaction norms") vary themselves due to genetic (or maternal) variation. The interactions at the population level characterize mean reaction norms of population size groups and of the particular populations within them. The interaction of maternal plant identity and environment is linked to differences in genotypic (or maternal) reaction norms, i.e. the sets of phenotypes that can be produced by maternal seed families exposed to different environments (Schlichting & Pigliucci, 1998).

Results

Pollination success and germination rates

We obtained 183 seed families out of 200 expected (10 populations \times 5 maternal plants \times 4 pollination treatments), since not all pollinations resulted in mature seeds. Differences in pollination

Table 2. Three orthogonal contrasts (C1, C2, C3) used to test specific hypotheses for the factor pollination treatment. The number of pollen donors in flowers exposed to free pollination (n) is unknown, but they probably originated from within the same populations as the maternal plants

Contrast	Pollination treatment				Test
	Free	Hand			
	(n pollen donors)	1 pollen donor	9 pollen donors		
	Within-population	Within-population	Within-population	Between-population	
C1	1	-1/3	-1/3	-1/3	Free vs. hand pollination
C2	0	1	-1/2	-1/2	1 vs. 9 pollen donors
C3	0	0	1	-1	Within vs. between population

success among pollination treatment groups and population size groups were significant, as discussed in Paschke et al. (2002b). Germination rates (arcsine-square-root proportion of germinated seeds of each seed family) tended to be higher in large than in small populations (ANOVA with population size group tested against random effect of population identity; $F_{1,8} = 3.82$, $p < 0.1$). From the 183 successful pollinations we obtained seedlings from 169 seed families (92%). From these 169 families, those in which ≥ 8 seeds germinated were kept for the greenhouse experiment. After applying this criterion, 117 seed families were used: 54 (out of 78 = 69%) in the small and 63 (out of 91 = 69%) in the large population size group. With respect to pollination, the low-diversity, within-population pollinations resulted in a final sample of 19 seed families (out of 36 = 52%), the high-diversity, within-population pollinations in 31 families (out of 43 = 72%), and the high-diversity, between-population pollinations in 24 families (out of 41 = 58%), and the free pollination in 43 families (out of 48 = 88%). The percentage of seeds that germinated for each family was entered as a covariate in the initial model for the analysis of the growth response of seedlings across experimental environments.

Effects of offspring environment

We found highly significant effects of offspring environment on all offspring traits (Table 3, row 2). Univariate analysis showed that these effects were mostly linear, indicating gradients of increasing stress (tables of univariate analysis not shown here). A significant time \times environment interaction in repeated-measures analysis (Table 3, row 17) indicated that the effects of the environmental treatments on the plants changed over time. For example, on day 120 leaves were smaller in the low-light than in the high-light environment, whereas on day 270 this difference was reversed. The relative ranking of environments as assessed by this plant character thus changed over time (Fig. 2).

Effects of small vs. large populations

Effects of the covariates were not consistent over time and had therefore no significant average effect (Table 3, row 1). Offspring from large and small populations did not differ significantly in any character in the repeated-measures analysis or in univariate ANOVAs (Table 3, row 3). However, variation among populations within population size

groups was highly significant for all offspring characters (Table 3, row 4). This reveals genetic variation among populations for the across-environment trait means (see also Paschke et al., 2003). Variation among maternal plants within populations was also highly significant (Table 3, row 5), suggesting genetic or maternal variation within populations for the across-environment trait means.

Environment \times population identity and environment \times maternal plant identity interactions

The interaction between offspring environment and population size group was generally small and not significant (Table 3, row 6, 21). However, the environment \times population identity interaction was significant for total plant size in the repeated-measures ANOVA (Table 3, row 7, 22). The time \times offspring environment \times maternal plant identity interaction significantly affected total plant size (Table 3, row 23), indicating that the differences among individual reaction norms of seed families, which were significant for early plant size on day 60 (in univariate ANOVA, $F_{38,1046} = 1.45$, $p < 0.05$), decreased with time during the experiment. The initial differences among reaction norms were therefore most likely due to maternal carry-over effects but were masked by population-identity effects.

Effects of pollination treatment and environment \times pollination treatment interactions

Effects of pollination treatment (averaged over populations, maternal plants and environments) were not significant in repeated measures analysis (Table 3, rows 9 and 24). However, in univariate ANOVAs two contrasts were significant ($p < 0.05$): hand pollination $>$ free pollination for total plant size on day 60 and high $>$ low pollen diversity within populations for leaf area on day 120 (Table 4). The interaction between pollination treatment and offspring environment was marginally significant in the repeated-measures ANOVA for leaf area (Table 3, row 25). When we split the pollination treatments into the three orthogonal contrasts described in Table 2 in univariate ANOVAs, we found that the interaction of the linear offspring environment and C3 (within- vs. between-population pollination) was significant for leaf area on day 120 ($F_{1,23} = 9.99$, $p < 0.005$) and final survival on day 300 ($F_{1,23} = 5.55$, $p < 0.05$): leaf area was larger

Table 3. Repeated-measures analysis for total plant size (= number leaves x plant height) of *Cochlearia bavarica* offspring from day 60, 120, 210 and 270 and leaf area from day 120 and day 210. Degrees of freedom are adjusted by Greenhouse–Geisser Epsilon for plant size; for leaf area this was not necessary because there were only two measurements. For further explanation see “Material and methods”

Source of variance	Total plant size (four dates)				Leaf area 120–270 days (day 120 vs. day 270)			
	df	MS	F	P <	df	MS	F	P <
<i>Between-subject effects</i>								
1. Covariates	3	16.7	0.01		1	3.1	1.06	
2. Offspring environment (= E)	3	40,594.6	48.80	0.005	3	37.2	19.88	0.005
3. Population size group (= S)	1	3,926.3	0.19		1	1.2	0.06	
4. Population identity (= pop.id.)	8	21,010.5	13.35	0.005	8	18.9	6.37	0.005
5. Maternal plant identity	36	1,573.9	1.89	0.005	36	3.0	1.59	0.01
6. E x S	3	179.3	0.10		3	1.4	0.43	
7. E x population identity	24	1,720.2	2.13	0.05	24	3.2	1.55	
8. E x maternal plant identity	107	805.8	0.97		107	2.1	1.11	
9. Pollination treatment	3	468.0	0.32		3	3.2	1.50	
10. E x pollination treatment	9	650.4	0.94		9	1.5	0.78	
11. S x pollination treatment	3	769.4	0.53		3	4.6	2.20	
12. Pop.id. x pollination treatment	23	1,460.3	1.76	0.005	23	2.1	1.13	
13. E x S x pollination treatment	9	1,004.9	1.45		9	3.3	1.78	0.10
14. E x pop.id. x pollination treat.	68	695.3	0.84		68	1.9	0.99	
15. Between-subjects residual	141	831.8			141	1.9		
<i>Within-subject effects</i>								
16. Time	2.5	136,456.1	69.29	0.005	1	1,611.8	226.69	0.005
17. Time x E	7.5	12,959.0	42.95	0.005	3	297.2	183.45	0.005
18. Time x S	2.5	1,269.7	0.64		1	1.9	0.26	
19. Time x population identity	20.0	1,969.3	3.76	0.005	8	7.1	5.60	0.005
20. Time x maternal plant identity	90.2	523.3	1.73	0.005	36	1.3	0.78	
21. Time x E x S	7.5	555.9	0.65		3	3.4	1.91	
22. Time x E x population identity	60.1	855.0	2.68	0.005	24	1.8	1.23	
23. Time x E x maternal plant identity	268.0	319.6	1.06	0.005	107	1.4	0.88	
24. Time x pollination treatment	7.5	501.5	1.43		3	2.0	0.75	
25. Time x E x pollination treatment	22.5	274.6	0.97		9	4.9	2.75	0.10
26. Time x S x pollination treatment	7.5	123.9	0.35		3	0.2	0.08	
27. Time x pop.id. x pollination treat.	57.6	350.6	1.16		23	2.6	1.61	0.05
28. Time x E x S x pollination treat.	22.5	314.0	1.11		9	2.1	1.18	
29. Time x E x pop.id. x poll.treat.	170.3	282.0	0.93		68	1.8	1.10	
30. Within-subject residual	354.0	301.7			141	1.6		

(Fig. 2) and survival of plants higher in less stressful environments for offspring obtained from between-population but not for offspring resulting from within-population pollination. While in line with the expectation that fertilization by pollen from outside the population leads to more vigorous progeny, this does not support the idea that a reduction of inbreeding depression should be stronger in more stressful environments. For total plant size on day 60 ($F_{2,54} = 8.35$, $p < 0.005$) and day 270 ($F_{2,47} = 4.63$, $p < 0.005$) and for leaf area on day 270 ($F_{2,47} = 3.60$, $p < 0.05$) the interaction environment x pollination C1 (free vs. hand-pollination) was significant, with offspring of free-

pollinated plants responding more strongly to different environments than offspring of hand-pollinated plants (Fig. 2).

The three-way interaction offspring environment x pollination treatment x population size group was significant for leaf area on day 270 (univariate ANOVA, $F_{3,22} = 3.08$, $p < 0.005$): under low light, offspring from small populations had larger leaf area when hand-pollinated between populations than when hand or freely pollinated within populations (Fig. 3, left panel). In large populations, the positive effect of pollen from outside the population was visible only in the low-water/low-light environment (Fig. 3, right panel).

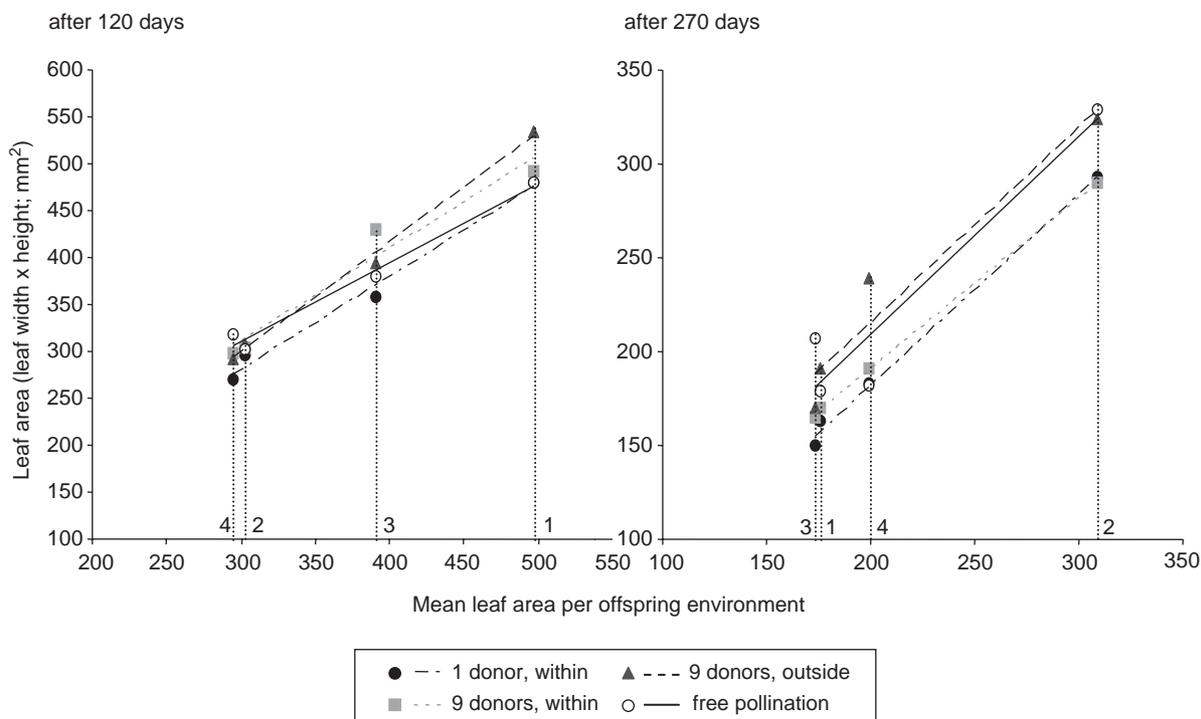


Figure 2. Growth response (leaf area, mm²) after 120 and 270 days of *Cochlearia bavarica* offspring derived from different pollination treatments and exposed to four experimental environments: high-water/high-light (= 1), high-water/low-light (= 2), low-water/high-light (= 3), low-water/low-light (= 4). Environments on the x-axis are ordered by ascending mean leaf area.

Table 4. Mean (\pm SE) plant size (cm), leaf area (cm²), and biomass (g) of offspring derived from different pollination treatments in *Cochlearia bavarica*. * differences among pollination treatments are significant ($P < 0.05$)

		1 pollen donor within-population	9 pollen donors within-population	9 pollen donors between-populations	Free pollination	
Plant size	Day 60	*	31.22 (0.88)	32.55 (0.69)	31.99 (0.85)	30.53 (0.57)
	Day 120		48.48 (1.85)	50.61 (1.22)	50.67 (1.51)	52.28 (1.05)
	Day 210		35.21 (1.43)	37.57 (1.03)	35.64 (1.22)	37.10 (0.95)
	Day 270		53.09 (2.20)	52.27 (1.51)	52.26 (1.82)	53.96 (1.41)
Leaf area	Day 120	*	3.49 (0.10)	3.82 (0.10)	3.86 (0.10)	3.71 (0.06)
	Day 270		1.97 (0.09)	2.04 (0.007)	2.27 (0.11)	2.22 (0.08)
Biomass	Day 270		0.163 (0.008)	0.166 (0.007)	0.181 (0.008)	0.167 (0.006)

Significant population identity x pollination treatment and time x population identity x pollination treatment interactions were observed for total plant size and leaf area, respectively, in repeated-measures ANOVA (Table 3, rows 12 and 27). In univariate ANOVA the corresponding significant interactions indicated that particular pollination treatments had different effects in different populations. Pollination with high pollen diversity had beneficial effects on offspring performance in some populations but not in others and pollination

between populations had positive effects in some populations but not in others.

Discussion

In this study, we investigated the effect of experimentally controlled environmental stress on offspring performance and the expression of inbreeding depression. Offspring were obtained from hand pollination with variable donor diversity and

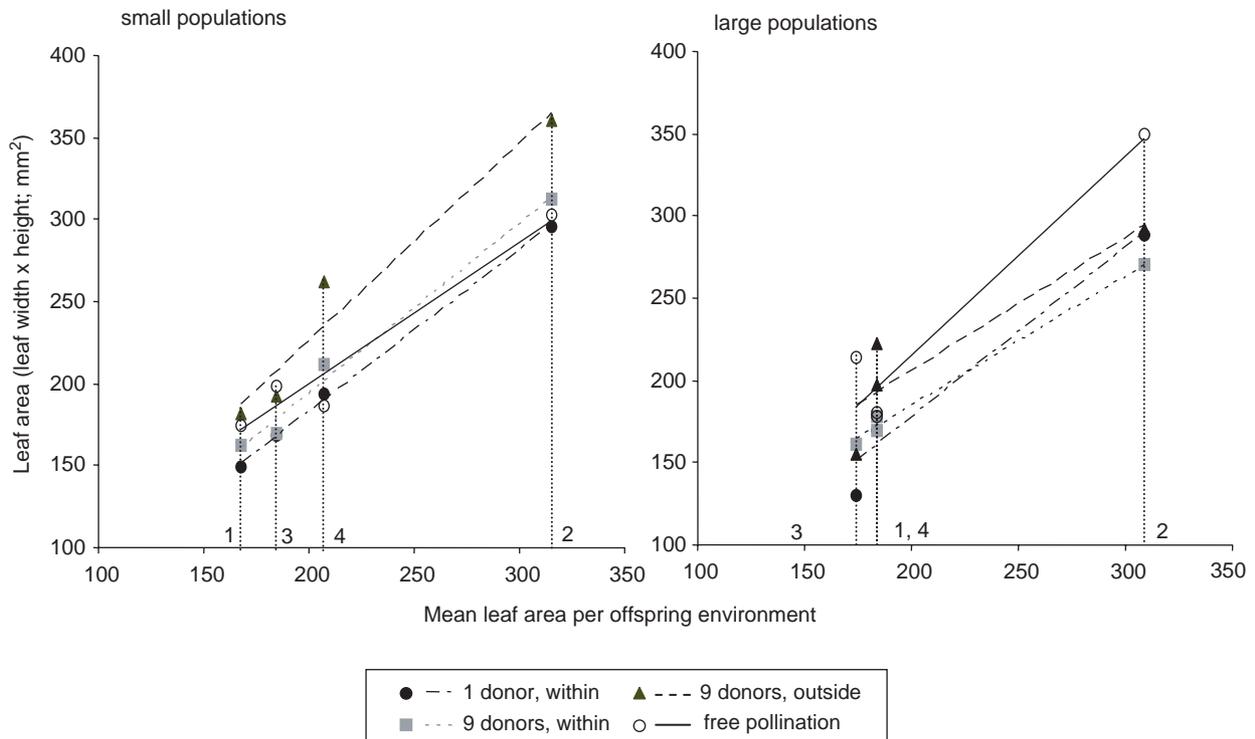


Figure 3. Growth response (leaf area after 270 days, mm²) of *Cochlearia bavarica* offspring originating from large and small populations and derived from different pollination treatments and exposed to four experimental environments: high-water/high-light (= 1), high-water/low-light (= 2), low water/high light (= 3), low water/low light (= 4). Environments on the x-axis are ordered by ascending mean leaf area.

provenance in small and large populations of the rare plant *C. bavarica* (Brassicaceae). First, we evaluate stressfulness of the experimental environments and ask if there is genetic variation for offspring traits in *C. bavarica*. We then discuss whether small populations benefit more from high pollen diversity or between-population pollination than large populations do, and whether these differences are more strongly expressed in stressful environments. We also discuss differences in pollination-treatment responses between individual populations and between maternal seed families within populations. Finally, we draw conclusions for conservation management of the species.

What are stressful environments for *Cochlearia bavarica*?

Stress can be empirically estimated by phenotypic responses of plants, either as stress symptoms likely to result in lower fitness (poor growth or reproduction; Tang & Turner, 1999; higher fluctuating asymmetry; Roy & Stanton, 1999) or stress-induced responses (e.g. induced changes in biomass

allocation; Bell & Sultan, 1999). In the present study we estimated stressfulness of the different environments indirectly by measuring plant growth performance (total plant size, leaf area) over time.

A significant effect of offspring environment on offspring characters showed phenotypic plasticity in the responses of *C. bavarica*. This implies that the environments were differently stressful to the plants. Based on the knowledge that *C. bavarica* only occurs at spring sites with continuous water supply and grows bigger in less shady sites, we assumed that stress should increase in the order high-water/high-light < high-water/low-light < low-water/high-light < low-water/low-light. In our experiment the high-water/low-light treatment was at some measurement times less stressful than the high-water/high-light treatment, indicating that low light was probably a less important stress factor than low water, perhaps because our experimental low-light condition was still lighter than the shade conditions in some natural growing sites of the species. Our results thus show that, to the plants, the stressfulness of the environments was not consistent over time and did not always affect the traits measured. This is in agreement with general results on phenotypic plasticity in

plants, whose expression can change seasonally, during ontogeny, with traits, with environment, etc. (Bradshaw, 1965; Sultan, 1987; Schmid, 1992). For example, seasonal changes could have been the reason for the temporal variation in perceived stressfulness of the environments (Paschke et al., 2003) and the high leaf area in the low light/high water environment on day 270 would be consistent with an allocational response to increased light capture (see, e.g. Rice & Bazzaz, 1989).

Is there genetic or maternal variation for offspring traits in *Cochlearia bavarica*?

Germination rate was significantly positively correlated with offspring performance: in families with high germination rate the offspring grew larger leaves. This may arise through maternal carry-over (e.g. as it may affect seed mass), or from different environmental conditions during germination (Schmid & Dolt, 1994). The latter however is unlikely since petri dishes with seeds of one family were treated equally and their position was re-randomized every three days. Germination rate was also marginally lower in small than in large populations, suggesting genetic differences between population size groups.

During the greenhouse experiment, we found significant effects of population identity and maternal plant identity within populations on offspring traits, indicating that part of the variation in offspring traits was due to genetic or maternal effects (Paschke et al., 2003).

Environment x population identity interactions were significant for most offspring traits, but only one environment x maternal plant identity interaction was significant (total plant size on day 60). The latter may reflect maternal carry-over on an early offspring trait. We therefore conclude that genetic variation for plastic response mainly occurred between, but not within populations. Rapid population differentiation of reaction norms across environmental gradients may be expected because of the combined action of local selection and random genetic drift in the predominantly small and isolated populations of *C. bavarica* (Schmid, 1984; Paschke et al., 2003). This view is consistent with the small within-population variation in reaction norms.

Effects of hand pollination versus free pollination

The performance of offspring derived from free pollination was, on average, lower than that of

offspring resulting from hand pollinations, but nevertheless it was slightly higher than the performance of offspring derived from within-population hand pollinations, particularly those with a single pollen donor. Probably a free-pollinated *C. bavarica* plant normally receives pollen from several donors (Paschke et al., 2002b). Despite the higher allelic diversity of large populations (Paschke et al., 2002a), their free-pollinated offspring only tended to have larger leaves but not generally higher performance than those from small populations. This suggests that the pollen a free-pollinated plant receives in a larger population is genetically not much more diverse than in a smaller population. Alternatively, differences may be expressed later in the life cycle or in traits not assessed in this study, e.g. reproductive traits of the offspring (Melser, Bijleveld, & Klinkhammer, 1999).

Effects of low vs. high pollen diversity

We expected that within-population hand pollination with low pollen diversity increases the level of biparental inbreeding, particularly in small populations, and that high pollen diversity decreases it. High pollen diversity can result in a positive sampling effect (Bernasconi et al., 2003, 2004): the more pollen donors there are, the more likely it is that a compatible, an unrelated, or a particularly "good" father will be among them and outcompete the other donors. As we could use only 41% of all seed families derived by hand pollination with low pollen diversity (vs. 70% of the seed families derived by pollination with high pollen diversity; see Methods), some inbreeding depression may have been purged before the start of the greenhouse experiment, leaving mainly seed families of high fitness. Nevertheless, within-population hand pollination with low pollen diversity had a significantly negative effect on later offspring performance in several populations (total plant size on day 210 and 270, dry mass on day 300). Similarly, in the previous study inbreeding depression decreased after germination, but increased again during later stages of offspring growth (Paschke et al., 2002b). If early selection is strong (e.g. at germination), inbreeding depression may be low in the immediately following stages, but become expressed later (Johnston & Schoen, 1996). While severe recessive deleterious mutations are more likely to be purged by inbreeding, mildly detrimental mutations can hardly be purged and may accumulate in later stages of offspring development (Husband & Schemske, 1996).

Because individual populations within population size groups varied in their response to pollen diversity, there was no overall indication of higher potential inbreeding depression in small than in large populations. There was also no significant difference in the response to environmental stress of offspring derived from pollination with low or high pollen diversity from either small or large populations. The negative effects of low pollen diversity on offspring performance were not significantly enhanced under stressful environmental conditions in our study. By comparison, other studies (Hauser & Loeschke, 1996; Dahlgaard & Loeschke, 1997) found stronger inbreeding depression under stressful environmental conditions. This suggests that even if purging has occurred under environmental conditions closer to the species' optimum, additional inbreeding depression is still expressed under novel conditions (Bijlsma et al., 1999). In the endemic *C. bavarica* with relatively short evolutionary history (Koch et al., 1996) and a total of ≤ 30 populations purging may not have occurred consistently or not more often in small than in large populations.

Effects of pollen from outside the population

Between-population hand pollination enhanced offspring performance. This suggests generally high inbreeding in populations of *C. bavarica*. If genetic diversity is low and relatedness between plants within a population is high, foreign pollen may increase heterozygosity and positively affect individual fitness. Positive effects after hand pollination with pollen from another population have been found in *Lychnis flos-cuculi* (Hauser & Loeschke, 1994). However, positive effects of between-population crosses may decay in later generations (Fenster & Dudash, 1994). In *C. bavarica*, the positive effect of foreign pollen was more pronounced in small than in large populations (see Fig. 3), suggesting that the lower allelic diversity of small populations (Paschke et al., 2002a) was correlated with lowered fitness-relevant genetic diversity. There was also an indication that the positive effect of "foreign" pollen was differentially expressed in different experimental environments. Again, between-population hand pollination did not increase offspring performance in the more stressful environments – rather, the opposite was true. In the case of *C. bavarica*, reduction of inbreeding and increased heterozygosity may reveal their positive effects on plant performance more strongly under beneficial than under stressful environmental conditions. As has been suggested

(Gebhardt-Henrich & Van Noordwijk, 1991; Schmid, 1992), expression of phenotypic and genetic variance may be limited in stressful environments. Thus, the hypothesis that inbreeding depression should be more severe under stressful conditions may require re-examination.

Conclusions

Hand pollination with low pollen diversity resulted in lower offspring growth performance. Between-population mating had beneficial effects especially under favorable environmental conditions and in small populations. *C. bavarica* is an endemic species. Its populations carry a relatively high genetic load and are threatened by inbreeding depression. Sufficient gene flow between populations seems especially important for such a species. Unfortunately, recent changes in the land-use pattern threaten the existing habitats. Conservation efforts for *C. bavarica* should therefore try to preserve genetic diversity within populations by increasing the size and connectivity of populations.

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