Inbreeding depression and heterosis vary in space and time in the serpentinophyte perennial *Minuartia smejkalii*

Inbrední deprese a heterózní efekt u hadcové vytrvalé rostliny *Minuartia smejkalii* se liší v prostoru a čase

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Heterosis has been used occasionally in attempts to save endangered populations of plants. However, there is a lack of knowledge on how inbreeding, heterosis and outbreeding depression jointly influence fitness of species with small, fragmented populations. Understanding the joint action of these processes is further complicated by variation among populations and environments in time and the stochastic effects of genetic drift. We assayed offspring of hand pollinated plants from three natural populations of *Minuartia smejkalii*, an endemic serpentinophyte perennial, in two soil treatments and three competitive environments for two consecutive years. We detected no early-acting inbreeding or outbreeding depression in a greenhouse. Late-acting inbreeding depression in the common garden was low in small populations and could be counteracted by heterosis. Both inbreeding depression and heterosis varied among traits, between years and with environmental stress. Notably inbreeding depression declined as environmental stress increased and phenotypic variation in the population decreased. Moreover, heterosis increased with environmental stress. Based on our results, we recommend a conservation approach in which between-population outbred plants are introduced into very small populations to maximise the benefits of heterosis in *M. smejkalii*. Nevertheless, extrapolation to other species, or even other populations of *M. smejkalii* of limited size, should be done with caution because of the stochastic effects of genetic drift that result in unique genetic consequences of outbreeding for each population.

Keywords: environment, inbreeding depression, genetic drift, heterosis, *Minuartia smejkalii*, outbreeding depression, serpentine, perennial

Introduction

In the current setting of human-mediated environmental degradation, plant and animal populations have become increasingly fragmented. Fragmentation decreases population size and connectivity, leading to limited mate availability and inbreeding, breeding among close relatives, or self-fertilization in hermaphrodites (Young et al. 1996, Bouzat 2010, Angeloni et al. 2011). Inbred offspring often suffer from inbreeding depression, that is, a relative decrease in the performance of inbred compared to outbred offspring. As inbreeding in a population increases, so does inbreeding depression, leading to a steady

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decline in offspring fitness (Liao & Reed 2009), reduced genetic diversity and population size, and if the population falls below a certain threshold level, to population extinction (extinction vortex, Gilpin & Soulé 1986). Although there is a possibility that inbred populations could be saved via purging of deleterious alleles (Leberg & Firmin 2008, Caballero et al. 2017), the conditions required for this are usually incompatible with the demography and genetics of small populations in which genetic drift results in a stochastic loss of heterozygosity, effectively preventing purging (Keller & Waller 2002).

Gene flow between populations can reduce the negative effects of inbreeding through heterosis, i.e. higher performance of between-population compared to within-population outbred offspring (Lynch & Walsh 1998). For heterosis to occur, the populations need to be sufficiently differentiated from one another so that the offspring benefit from gene complementation or overdominance (Escobar et al. 2008). However, natural gene flow is also limited as a consequence of population fragmentation and isolation by anthropogenic barriers (Kwak et al. 1998, Luijten et al. 2002, Honnay & Jacquemyn 2007). Conservation biologists can rescue small, isolated populations by purposefully introducing foreign individuals, but this practice entails its own risks. In particular, outbreeding depression due to the introduction of locally non-adapted genotypes or the breakdown of adaptive genetic complexes can counteract the benefits of heterosis (Edmands 1999, 2007). Despite having been known for a long time and empirically demonstrated in experimental populations and used practically to enhance crop production, examples of the use of heterosis for the genetic rescue of endangered populations are still very scarce (Edmands 2007, Frankham 2015).

Obtaining a precise image of the interplay between inbreeding, heterosis, outbreeding and how they shape population genetic structure and fitness is further complicated by their context-dependence. Inbreeding depression is expected to be higher in predominantly outcrossing than predominantly selfing populations, because of the more effective purging of deleterious alleles in selfing populations, although exceptions are regularly documented (Byers & Waller 1999, Winn et al. 2011). Environment-dependence of inbreeding depression is extensively documented and yet there is still no consensus about how environmental stress affects inbreeding depression (Armbruster & Reed 2005, Waller et al. 2008, Reed et al. 2012, Sandner & Matthies 2016). More recent studies show that a better predictor of inbreeding depression is population evolutionary potential measured in terms of the amount of phenotypic variation (Waller et al. 2008, Sandner & Matthies 2016). Inbreeding depression also varies substantially among species, populations and lineages (Armbruster & Reed 2005, Escobar et al. 2008, Bouzat 2010), among traits (Husband & Schemske 1996, Winn et al. 2011) and over time (Johnston 1992, Montalvo 1994, Sandner & Matthies 2016, Nickolas et al. 2019). Even less is known about the variation in heterosis and outbreeding depression since they have not been systematically studied. However, context-dependent heterosis is more often observed than not (Waser et al. 2000, Escobar et al. 2008, Wagenius et al. 2010, Prill et al. 2014, Sandner & Matthies 2016, Nickolas et al. 2019). Finally, because of the stochastic effects of genetic drift, it is plausible that in small, isolated populations, no reliable predictions can be made about the extent of inbreeding and outbreeding depression. Each population evolving under genetic drift has a unique genetic structure that is at least partly independent of the environment in which it has evolved (Bouzat 2010). Altogether, these results render the task of conservation biologists and managers extremely complicated. Increasing our knowledge of trends in variation in inbreeding depression, heterosis and outbreeding depression across environments, populations, and over time can provide generalizable results and facilitate the tasks of conservation biologists.

Minuartia smejkalii is an endemic perennial confined exclusively to small serpentine outcrops in the central part of the Czech Republic. Being an obligate serpentinophyte, its populations are naturally highly fragmented because of the patchy distribution of serpentine soils. Anthropogenic actions have further reduced the size and fragmentation of the populations since the 1960s–1970s. Currently, there are seven natural populations of *M. smejkalii* in two regions in the Czech Republic, with a total of about 1200 individuals. The populations have high genetic diversity and low population differentiation. It is likely, however, that this is only a transient state following a recent bottleneck and it is expected that in time the species will lose genetic diversity and inbreeding will increase unless population sizes are increased (Stojanova et al. 2020). The highly fragmented distribution renders the populations particularly susceptible to genetic drift and inbreeding. This can in turn enhance inbreeding depression within populations, but it also can result in heterosis if populations are mixed. On the other hand, the species is adapted for survival in the specific edaphic conditions of serpentine soils, which are high in toxins, poor in nutrients and prone to drought, but with low levels of interspecific competition. If the extant populations have adapted locally to these harsh environmental conditions, between-population breeding could result in outbreeding depression.

To help develop the most efficient conservation program for this species, it is important to know the extent of inbreeding depression and whether the balance between heterosis and outbreeding depression can outweigh inbreeding depression in different settings. Despite recent attempts to compile knowledge on the benefit of heterosis for conservation purposes (Edmands 2007, Frankham et al. 2011, Frankham 2015), such data are scarce. This could explain why no clear-cut conservation recommendations are available regarding the use of heterosis for conservation purposes (Sutherland et al. 2019).

In this paper, we studied offspring produced by three types of controlled crosses in two different soils, three experimental treatments and over two consecutive years. We measured traits at different stages of the lifecycle in order to answer the following questions: (i) Is there inbreeding depression, heterosis, and outbreeding depression in *M. smejkalii*? (ii) How do they vary among populations, environments and over time? More generally, (iii) can heterosis compensate for the negative effects of inbreeding and outbreeding depression across various conditions? (iv) Based on all the above observations can we devise a general conservation plan for this species?

Material and methods

Study species and plant material

Minuartia smejkalii is a short-lived perennial, with some individuals in natural populations persisting for up to 18 years. Plants attain reproductive maturity one to two years after germination. Although selfing rates in *M. smejkalii* have not been estimated, its protogynous flowers and the patterns of neutral genetic diversity (high within- and low between-population diversity) indicate a mixed to predominantly outcrossing mating system (Stojanova et al. 2020). Pollination biology of the species has not been studied, but field and common garden observations have recorded visits from *Syrphidae* and bees of the genus *Bombus* (Pánková, personal observation). The seeds of *M. smejkalii* have a surface area of about 2.5 mm^2, are devoid of dispersal structures and their average free dispersal distance is 21.6±0.03 cm according to simulations, but they could be dispersed over longer distances by ants (Lozada-Gobilard et al. 2020).

At present, *M. smejkalii* is found in two areas in the Czech Republic, the National Nature Monument and a Site of Community Importance Želivka and the Nature Monument and a Site of Community Importance Hrnčíře. There are six populations of *M. smejkalii* at Želivka separated by 100–1800 m from one another, and one population at Hrnčíře. For this study, seeds from three populations in the Želivka region were harvested (the remaining populations at Želivka and Hrnčíře did not produce enough seed at the time of the harvest). Description of the populations and their population genetic parameters are in Table 1.

Table 1. – *Minuartia smejkalii* populations included in this study. Number of individuals in the population = total number of flowering individuals in the natural population at the time of sampling. Number of individuals sampled = number of individuals from which seed was harvested and then grown as the parental generation in a greenhouse. Pollen donors = average number of pollen donors per individual; S.E. = standard error. Bold values correspond to within-population crosses. Population Z6 had fewer pollen donors because of the lower flowering rate of plants grown in pots in 2017. H_{obs} – observed heterozygosity, H_{exp} – expected heterozygosity, F_{IS} – Fisher's fixation index, %nonHW – percentage of loci that are not in Hardy-Weinberg equilibrium. Population genetic data adapted from Stojanova et al. (2020).

	Number of individuals		Pollen donors ± S.E.			Population genetics			Distance from (in m)		
Population	In the population	Sampled	Z1	Z2	Z6	H_{obs}	H_{exp}	F_{IS}	Z1	Z2	Z6
Z1	7	5	**14.7±0.7**	11.8±1.0	8.2±1.9	0.210	0.192	–0.095	–	200	1800
Z2	255	40	8.7±1.4	**9.0±1.1**	8.4±1.7	0.259	0.217	–0.193	200	–	1750
Z6	53	7	3.2±0.7	3.1±0.6	**4.0±0. 5**	0.213	0.208	–0.027	1800	1750	–

Seeds were harvested in June 2016 and stored in a dry, dark place. In late autumn 2016, the seeds were germinated in 47 × 20 × 10 cm trays filled with serpentine soil, which were placed in a heated greenhouse (minimal daily temperature 10 °C) of the Botanical Institute of the Czech Academy of Science (49°59'37.9"N, 14°33'57.8"E). About six months after germination, the seedlings were each planted in a 10 × 10 cm pot filled with serpentine soil and placed outdoors. When at least 30% of the plants were flowering, five to 10 plants per population were designated as pollen recipients.

Controlled crosses

Controlled crosses were made in August and September 2017, in a heated greenhouse. Crossed plants were kept in 120 × 80 × 60 cm cages covered with a double layer of fine mesh fabric, which allowed light to enter and wind to pass through the cage. Pollinations were performed daily between 8 am and 12 noon. The pollinated flowers were castrated one to three days before pollen deposition, when their anthers were non-dehisced and the stigma was unreceptive, thus avoiding self-pollination.

Each pollen recipient was pollinated in one of four ways: pollen from the same plant (inbreeding); pollen from the same population as the pollen donor (outbreeding within a population); and pollen from each of the two other populations as donors (outbreeding between populations). Each of the 939 pollinated flowers was marked with tape bearing a unique code. There was no need to prevent post-pollination contamination because intact castrated flowers did not develop seeds (B. Stojanova, personal observation). Mature capsules were collected three weeks after pollination and stored in paper bags in a dry place at room temperature until the seeds were counted. The seeds were then placed in Eppendorf tubes with small holes for air circulation and stored in dark, dry conditions until used in the germination assays.

Seed germination

Germination assays started in March 2018, in the same greenhouse as used above. Thus, if the fresh seeds were dormant then dormancy should have been broken by after ripening during dry storage. Because the number of seeds varied greatly between flowers, full-sib families (from a single flower) of six or more seeds were sown in individual pots, whereas full-sib families with less than six seeds were pooled by maternal plant and pollination type (half-sib families) and sown together. For each full- and half-sib family, six to ten seeds were used in the germination assays. Half of the seeds were germinated in serpentine soil and the other half in regular garden (control) soil, resulting in 1224 and 1247 seeds, respectively. The number of seedlings was first assayed on 26 April 2018, about three weeks after the seeds were sown, and again prior to seedling transplantation (24 May 2018).

Experimental treatments of plants

The seedlings were planted in individual 10 × 10 × 10 cm pots containing the same kind of soil as they were germinated in. The pots were placed in a common garden and distributed as equally as possible by soil type and cross type across three treatments: competition, shade or control. In the competition treatment, two ramets of *Festuca ovina* were planted in each pot. In the shade treatment, the plants were placed under a canopy of mesh fabric that reduced sunlight intensity by 70%. In the control treatment, plants were grown without shade and without a competing species. We designated the garden soil-control treatment as the standard environment against which plant performance was compared in subsequent analyses. This treatment should be the least stressful for *M. smejkalii*, as the species restriction to serpentine soils is most likely due to its tendency to avoid interspecific competition rather than a preference for the specific edaphic conditions associated with serpentine soil (Harrison & Rajakaruna 2011). There were six soil-treatment combinations: garden soil-control (202 plants), serpentine-control (154), garden-competition (200), serpentine-competition (155), garden-shade (90) and serpentine-shade (45). The experimental design was unbalanced because of the lower percentage germination in serpentine soil.

Trait measurement

We classified the traits measured based on their occurrence during the life cycle as either: early, mid or late traits (Husband & Schemske 1996). The early trait pollination success was estimated as the number of mature seeds per capsule, including those flowers that were not successfully pollinated (i.e. none mature seeds). We also analysed separately seed production in successfully pollinated flowers in terms of the number of seeds (in plants with at least one mature seed). The mid trait, percentage germination, was assessed as the number of seedlings alive at transplantation relative to the number of seeds sown. Late traits were assayed in two consecutive years (2018 and 2019) in late June / early July. For each date, we measured tussock width and counted the number of flowering and non-flowering ramets and the number of flowers on up to ten flowering ramets. Based on these measurements, we calculated the total number of ramets (sum of flowering and non-flowering ramets) and flower set (average number of flowers per ramet multiplied by the number of flowering ramets). Plant mortality between 2018 and 2019 was also recorded.

Data analysis

All analyses were performed in R 3.5.2 (R Core Team 2014). All non-binary dependent variables were log (x + 1) transformed to produce additive ratio estimates (Johnston & Schoen 1994). All models contained cross type, population, their interaction, dam (and sire when relevant) nested in population. For germination, the model also included type of soil and its interactions, and for late traits it also included experimental treatment, year of measurement and all of their interactions.

We tested the effect of sire in a model with full-sib families only and compared its output with a model fitted to the full dataset with the same variables except sire. Including sire did not substantially modify the conclusions (compare Electronic Appendix 1–2); thus, the full dataset was used in subsequent analyses. All independent variables were declared as fixed to avoid convergence and singularity fit problems that occured in some mixed-effects models. For the same reason, when zero-inflated data were analysed using logistic models some higher-level interactions could not be fitted. When a significant interaction between two or more dependent variables was detected, the data were analysed separately using the factor levels of one of the variables. The validity of all models was checked by visually inspecting the residuals versus fitted values of plots. With exception of the zero-inflated variables, all models satisfied the assumptions regarding residuals, homoscedasticity and normal distribution, reasonably well.

Several traits had zero-inflated distributions. For pollination success, the zero-inflation could be due to weather conditions during pollination and effect of the experimenter. To account for this, we first obtained the residuals of a Poisson regression of seed number with pollination date as an independent variable and then analysed the residuals using linear regression. Flowering traits in the first year also had a zero-inflated distribution, thus we analysed them by fitting two models: a logistic regression, whose coefficients correspond to the odds ratio that a plant will start flowering, and a linear regression of the log-transformed data subset of plants that produced at least one flower, which is informative in terms of the differences in number of flowers among populations, treatments and cross types.

Percentage mortality between 2018 and 2019 was analysed using logistic regression with cross type, population, treatment, soil and all their two-way interactions.

Inbreeding and outbreeding depression

Coefficients of classical population-level inbreeding depression were estimated as

$$\Delta_{ID} = 1 - \left(w_{self} / w_{WPO} \right)$$

with w_{self} and w_{WPO} being the fitness of inbred and within-population outbred offspring, respectively. If inbreeding depression was negative (inbreeding benefit), we used

$$\Delta_{ID} = \left(w_{WPO} / w_{self} \right) - 1$$

to keep the variation of the estimates between –1 and 1 (Ågren & Schemske 1993). We estimated outbreeding depression as

$$\Delta_{OD} = 1 - \left(w_{WPO} / w_{BPO} \right)$$

and heterosis as

$$\Delta_{OD} = \left(w_{BPO} / w_{WPO} \right) - 1,$$

with w_{BPO} being the fitness of between-population outbred offspring.

We could not calculate family-based estimates of inbreeding depression and its variance because maternal lines were highly unbalanced. Therefore, we calculated the change in log fitness of inbred individuals relative to the fitness of within-population outbred individuals (Keller & Waller 2002) based on linear regression coefficients:

$$\ln\left(\Delta w_{ID}\right) = \ln\left(w_{WPO}\right) - \ln\left(w_{self}\right)$$

Similarly, we calculated the change in log fitness of between-population outbred individuals relative to within-population outbred individuals as

$$\ln\left(\Delta w_{OD}\right) = \ln\left(w_{WPO}\right) - \ln\left(w_{BPO}\right)$$

The regression coefficients were obtained from linear regressions for each population – year combination with cross type, soil (for mid and late traits), treatment (for late traits), and their interactions as explanatory variables. For the variable cross type, the level within-population outbreeding was always set as the intercept. Depending on whether the regression tested for inbreeding or outbreeding effects, this variable had the level of inbreeding (for estimates of inbreeding depression) or the two levels of between-population outbreeding (for outbreeding depression). If a significant effect between cross type and soil or treatment was detected, indicating the presence of environment-dependent effects, the data were further split by soil, treatment, or soil x treatment to estimate inbreeding and outbreeding depression in each treatment. When cross type had a significant effect, the significance of the inbreeding (or outbreeding) coefficient was tested using a t-test. Explicit testing for an association between inbreeding and outbreeding depression, and plant mortality was not possible because of the occurrence of 0 or 1 probabilities in the model, which inflates the parameter estimates. We instead report and compare mortality for each population.

To compare our results with those in the literature (Husband & Schemske 1996, Winn et al. 2011), we calculated three-stage cumulative inbreeding and outbreeding depression following Husband and Schemske (1996):

$$1-\left(\frac{w_{self}^{early}}{w_{WPO}^{early}}\right)\times\left(\frac{w_{self}^{mid}}{w_{WPO}^{mid}}\right)\times\left(\frac{w_{self}^{late}}{w_{WPO}^{late}}\right)$$

for inbreeding depression and

$$1-\left(\frac{w_{BPO}^{early}}{w_{WPO}^{early}}\right)\times\left(\frac{w_{BPO}^{mid}}{w_{WPO}^{mid}}\right)\times\left(\frac{w_{BPO}^{late}}{w_{WPO}^{late}}\right),$$

for outbreeding depression. Note that with this formula if there is a cumulative inbreeding benefit or heterosis, the estimate can be higher than –1, because the fitness ratios are not adjusted to be always contained between –1 and 0. Because we measured two early and three late traits, we estimated cumulative inbreeding depression for all combinations of one early, one mid and one late trait. This resulted in a total of six estimates of cumulative inbreeding depression per population.

To test whether late-acting inbreeding and outbreeding depression were affected by environmental stress or the coefficient of phenotypic variation, we used a partial regression method similar to that described by Sandner and Matthies (2016). First, we calculated environmental stress as

$$\Delta_E = 1-\left(\frac{\mu_{env}}{\mu_{ctrl}}\right)$$

With μ_{env} and μ_{ctr} being the trait mean for within-population outbred offspring in the measured environment and in the control-garden soil treatment, respectively. A positive coefficient for environmental stress indicates higher stress in the measured environment than in the control-garden treatment and a negative coefficient indicates lower stress in the measured environment. The phenotypic variation, which is an estimate of the opportunity for selection (Waller et al. 2008), was calculated as the coefficient of variation squared

$$CV^2 = \mathrm{var}_{env} / \mu_{env}^2$$

for each cross type separately and then averaged to obtain the mean coefficient of variation for a trait in a given population, year and environment. Then we used partial regression to examine the relationship between inbreeding/outbreeding depression, environmental stress and the coefficient of variation (Moya-Laraño & Corcobado 2008). We also tested whether the change in the sign of inbreeding depression across different environments coincides with the change in the sign of the coefficient of variation. We estimated the changes in the sign of inbreeding/outbreeding depression and coefficient of variation relative to the control treatment-garden soil environment for each trait in each population and year. Then we counted the occurrence of matching changes in the signs between inbreeding/outbreeding depression and coefficient of variation. Finally, we calculated the binomial probability to obtain the number of matching cases. A significant result indicates a positive association between inbreeding/outbreeding depression and coefficient of variation.

Results

The estimates of inbreeding/outbreeding depression and changes in log fitness were very similar (compare Electronic Appendices 3–5 with estimates reported in the main text). We therefore report the classical estimates of inbreeding and outbreeding depression combined with the P-values obtained from the estimates of the change in log fitness.

Early traits

After accounting for the effect of date of pollination, pollination success did not differ significantly between cross types and populations (Electronic Appendices 6, 8, Fig. 1). For number of seeds, we detected significant cross type x population interaction due to the presence of inbreeding depression in Z6. We detected no significant early-acting outbreeding depression.

Mid trait

Percentage germination was significantly higher in garden than in serpentine soil (Electronic Appendices 6, 7). Germination differed significantly between cross types, but not between populations (Fig. 1). We observed inbreeding depression in the model containing all populations, but when tested by population, we only found a significant effect of cross type x soil for population Z1 (Fig. 1.A, Electronic Appendix 8). Specifically, for germination in serpentine soil we detected strong and significant inbreeding depression ($\Delta_{\text{ID serpentine}} = 0.469$), whereas in garden soil there was a (non-significant) inbreeding benefit ($\Delta_{\text{ID garden}} = -0.017$). We detected no significant outbreeding depression for germination.

Late traits

Late traits were analysed separately by year and population since these two dependent variables were part of most significant higher-level interactions (Electronic Appendices 1, 9). Inbred offspring generally performed less well than those from outcrossed plants, although we did not detect significant inbreeding depression in population Z6 for all but one trait in the second year (Fig. 2, Electronic Appendix 10). Conversely, inbreeding depression was significant for almost all traits in Z1 and Z2. In general inbreeding depression was higher in Z2 than in Z1, especially in 2018.

In Z1 and Z2 we detected significant environment-dependent inbreeding depression for some traits (Fig. 3, Electronic Appendix 10). In Z1, stem number (Fig. 3A) and flower set in 2019 (Fig. 3B) showed high inbreeding depression in the less stressful, competition treatment and inbreeding benefit in the more stressful, shade treatment (environmental stress estimates ranged from 0.30 to 0.91, depending on year and soil type). In population Z2 inbreeding depression in terms of stem number in 2018 was highest in the shade, intermediate in the control and lowest in the competition treatment (Fig. 3C). In population Z2 in 2019 plant width also showed significant inbreeding depression in the control, but not in the competition or shade treatments (Fig. 3D).

Overall inbreeding depression decreased as environmental stress increased (partial regression coefficient –0.242, $P < 0.001$). The quantitative relationship between inbreeding depression and the coefficient of variation (partial regression coefficient –0.034, $P = 0.474$, Electronic Appendix 12) was not significant, however, the quantitative association

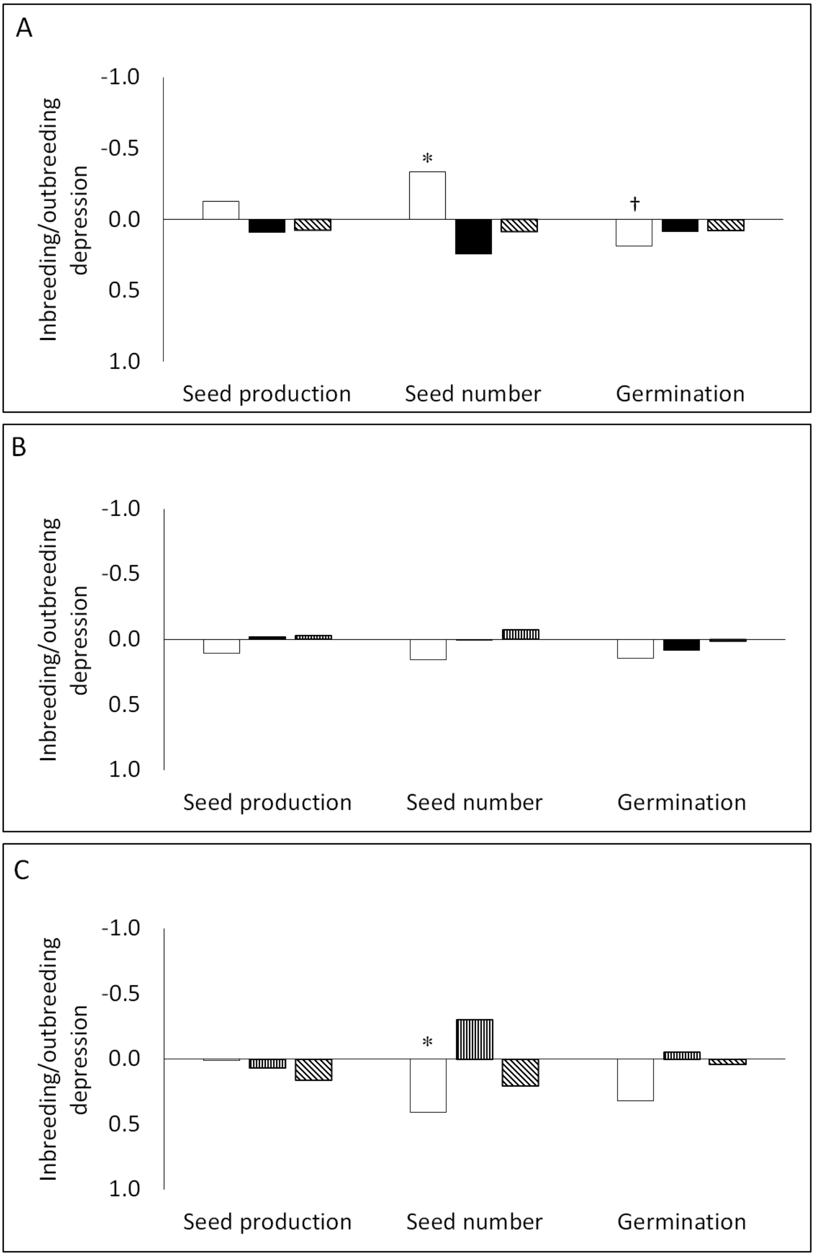

Fig. 1. – Estimates of inbreeding and outbreeding depression for early and mid-cycle traits in population Z1 (A), Z2 (B) and Z6 (C). The bars correspond to the performance of inbred or between-population outbred offspring relative to that of the within-population outbred offspring (set at 0). Empty bars – inbred, vertically striped bars – outbred with Z1 as pollen donor, diagonally striped bars – outbred with Z2, full bars – outbred with Z6; * – estimates with significant effect of cross type, † – estimates with significant interaction cross type × soil (see details in main text). Note the inversion of the y-axis, so that values below the axis indicate inbreeding/outbreeding depression, and values above inbreeding benefit or heterosis.

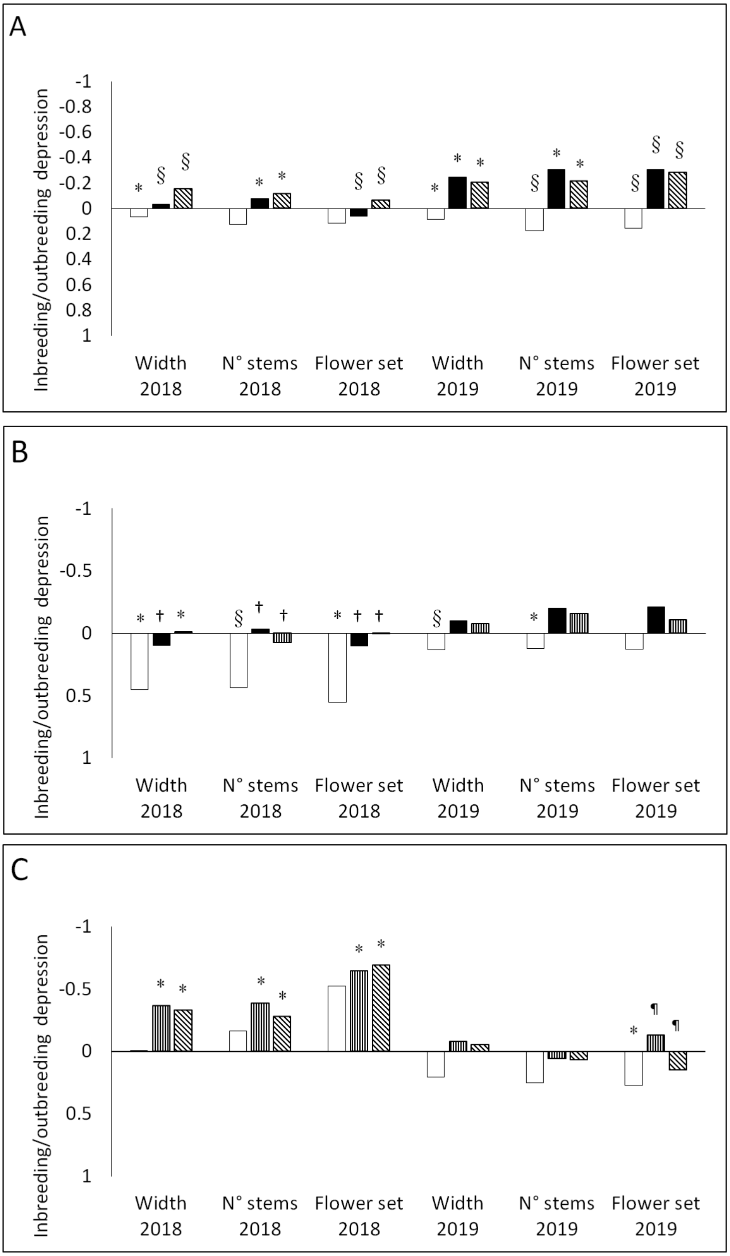

Fig. 2. – Estimates of inbreeding and outbreeding depression. The bars correspond to the performance of inbred or between-population outbred offspring relative to that of the within-population outbred offspring (set at 0). Empty bars – inbred, vertically striped bars – outbred with Z1 as pollen donor, diagonally striped bars – outbred with Z2, full bars – outbred with Z6; * – estimates with significant effect of cross type, § – estimates with significant interaction of cross type × treatment, † – estimates with significant interaction of cross type × soil, ¶ – estimates with significant interaction cross type × soil × treatment. Note the inversion of the y-axis, so that values below the axis indicate inbreeding/outbreeding depression, and values above inbreeding benefit or heterosis.

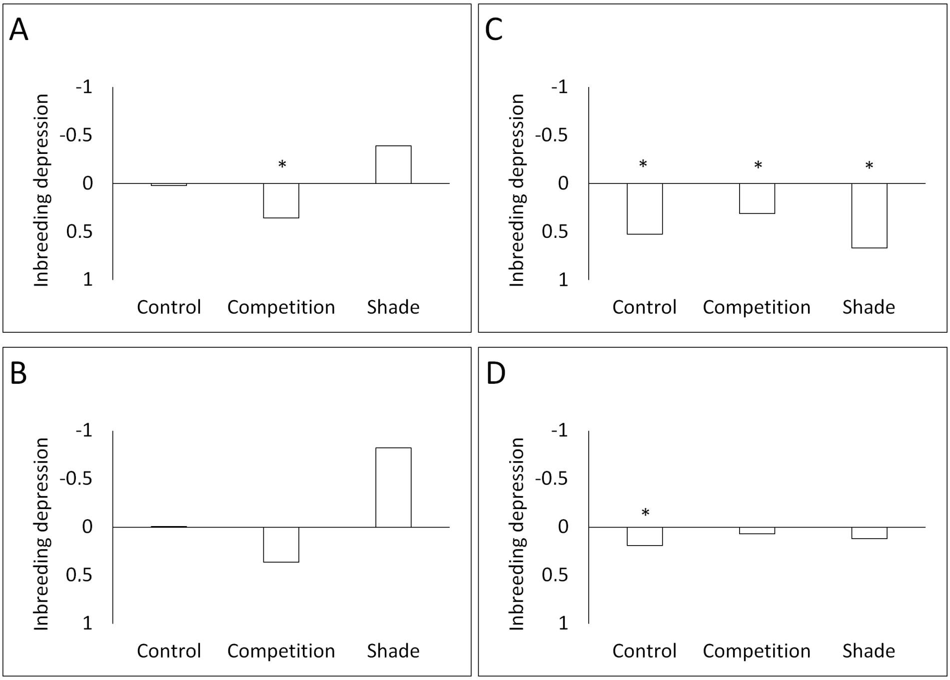

Fig. 3. – Treatment-dependent inbreeding depression in populations Z1 and Z2. (A) Number of stems in Z1 in 2019, (B) flower set in Z1 in 2019, (C) number of stems in Z2 in 2018, (D) plant width in Z2 in 2019. Stars mark values that differ significantly from the intercept (set as mean value of trait for within-population outcrossed offspring).

between inbreeding depression and the coefficient of variation, 22 out of 70 cases, indicated a coincidence in the change in sign between phenotypic variation and inbreeding depression (binomial expansion probability: $P \leq 0.0012$).

Between-population outbreeding generally resulted in heterosis, although the increase in log fitness was not always significant (Fig. 2, Electronic Appendix 10). Highest heterosis was recorded for Z6 in 2018 for all traits. In 2019, heterosis for Z6 was lower and only significant for flower set (Fig. 2C). For Z1, heterosis was considerably lower than for Z6 in 2018, but higher in 2019 (Fig. 2A). For Z2, significant outbreeding depression was detected in 2018, and heterosis in 2019 (Fig. 2B).

We detected significant environment-dependent outbreeding depression or heterosis in at least one trait in each population (Electronic Appendix 10, Fig. 4). For Z1 in 2018, outbreeding depression adversely affected flower set and plant width in the shade and heterosis benefitted the control and competition treatments (Fig. 4A and B). In 2019, only flower set showed environment-dependent heterosis, and unlike in 2018 the plants benefitted from heterosis in the shade treatment (Fig. 4C). In Z2 in 2018, outbreeding depression affected all traits in serpentine and heterosis in garden soil (Fig. 4D–F), but no environment-dependent effects were detected in 2019. In Z6, both soil and experimental treatments had an effect on flower set in 2019. In particular, high heterosis for offspring sired by Z1 and grown in serpentine soil was recorded (Fig. 4G).

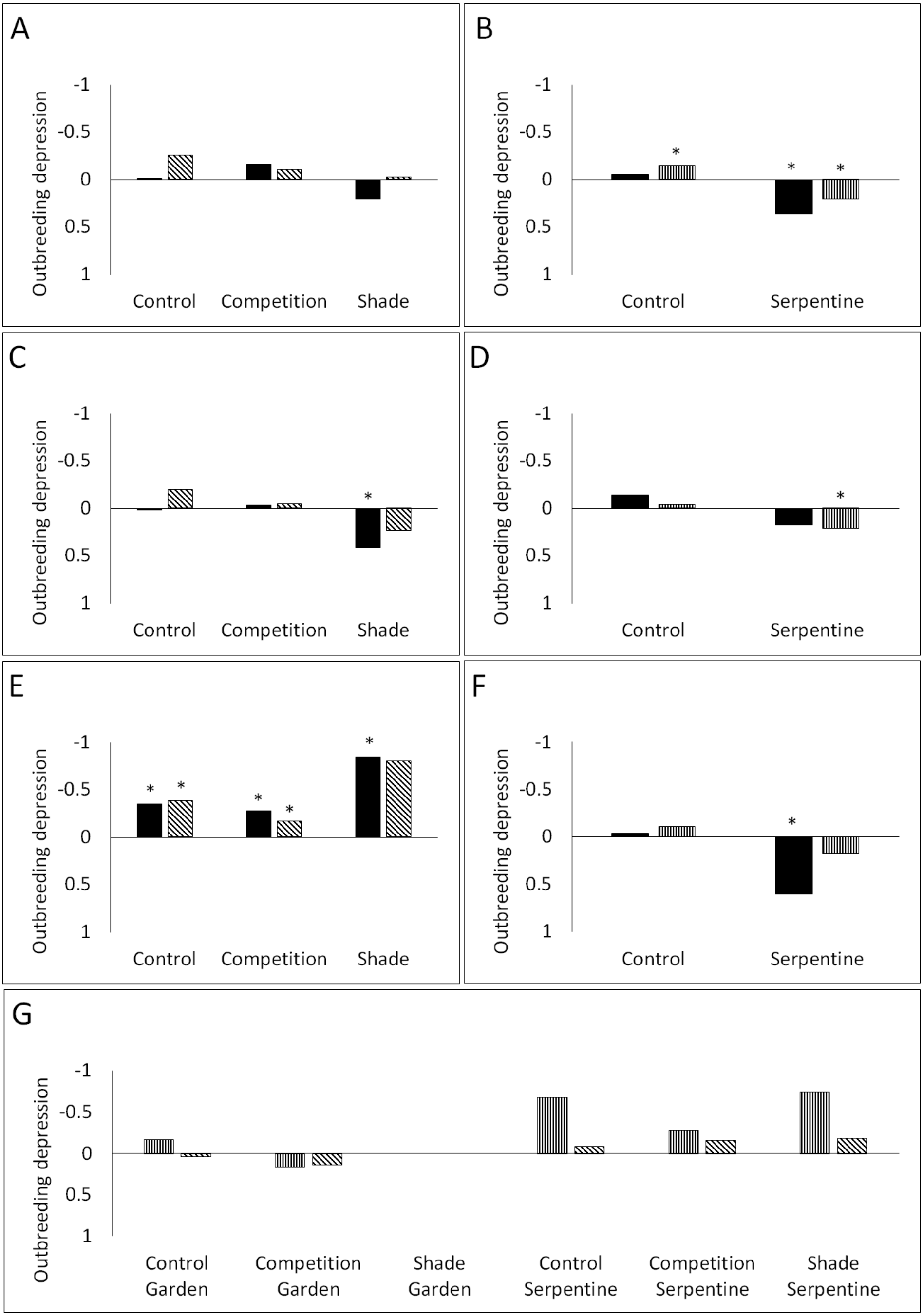

Fig. 4. – Treatment-dependent outbreeding depression in Z1 (A, C, E), soil-dependent outbreeding depression in Z2 (B, D, F) and treatment × soil-dependent outbreeding depression in Z6 (G). (A) plant width in 2018 in Z1, (C) flower set in 2018 in Z1, (E) flower set in 2019 in Z1, (B) plant width in 2018 in Z2, (D) number of stems in 2018 in Z2, (F) flower set in 2018 in Z2, (G) flower set in 2019 in Z6. Empty bars – inbred, vertically striped bars – outbred with Z1 as pollen donor, diagonally striped bars – outbred with Z2, full bars – outbred with Z6; * – estimates that differ significantly from the intercept (set as mean value of trait for within-population outcrossed offspring).

Table 2. – Analyses of plant mortality between 2018 and 2019 and percentage mortality per cross type for each population. Significant values are in bold. Sample sizes are next to the name of the population. Colour code corresponds to the percentage mortality recorded in each population: dark grey – high mortality, white – low mortality.

	Z1 (392)			Z2 (292)			Z6 (184)		
	Df	Deviance	P-value	Df	Deviance	P-value	Df	Deviance	P-value
Treatment	2	170.06	**< 0.001**	2	71.07	0.072	2	120.41	**< 0.001**
Soil	1	152.08	0.636	1	66.62	0.369	1	99.15	0.077
Cross type	3	161.81	**0.019**	3	67.26	0.696	3	104.7	**0.034**
Treatment x Soil	2	122.78	0.468	2	54.37	0.079	2	86.35	0.146
Treatment x Cross type	6	141.94	**0.002**	6	53.73	0.619	5	88.65	0.291
Soil x Cross type	3	129.8	**0.036**	3	49.30	1.000	3	86.34	0.279
% mortality									
Inbred		12.8			4.1			7.7	
WP Outbred		2.2			1.4			14.3	
BP Outbred	Z6	4.9		Z6	3.3		Z1	2.1	
BP Outbred	Z2	4.7		Z1	2.2		Z2	17.4	

We detected no significant effect of environmental stress or CV^2 on outbreeding depression (partial regression coefficients: –0.033, P = 0.565; and 0.0001, P = 0.925, respectively, Electronic Appendix 12), although the qualitative association between outbreeding depression and phenotypic variation was significant (110 of 180 matching cases, P ≤ 0.002).

Although explicit testing for inbreeding and outbreeding depression on plant mortality was not possible, the trait varied significantly among different cross types in population Z1 and Z6, but not in population Z2 (Table 2, Electronic Appendix 11). Mortality of inbred offspring compared to within-population outbred offspring was six-fold higher in population Z1 and three-fold higher in population Z2, which indicates inbreeding depression. Inversely, mortality was lower in inbred relative to outbred offspring in Z6, which indicates that inbreeding was beneficial. Mortality was higher in all but one case of between-population outbred offspring relative to within-population outbred offspring, indicating outbreeding depression.

Cumulative inbreeding depression

Cumulative estimates of inbreeding depression based on three life stages were similar regardless of the early and late traits used for the estimates (Electronic Appendix 14). Cumulative inbreeding depression was high (over 0.5) in all three populations (Fig. 5). Estimates of cumulative heterosis were more variable for different early and late trait combinations, but on average the effect of between-population breeding on plant fitness in each population was positive (Electronic Appendix 14). Population Z6 had the highest cumulative heterosis and population Z1 the lowest (Fig. 5).

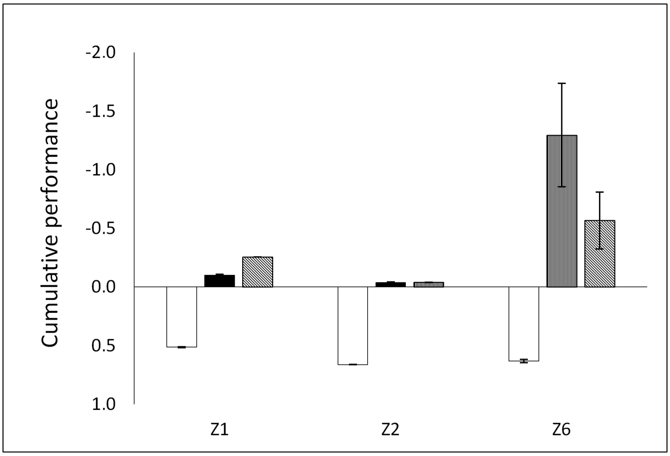

Fig. 5. – Average estimates of cumulative of inbreeding depression and heterosis per population. The bars correspond to the average cumulative performance of six estimates for different combinations of early and late-cycle traits. Empty bars – inbred, vertically striped bars – outbred with Z1 as pollen donor, diagonally striped bars – outbred with Z2, black – outbred with Z6. Note the inversion of the y-axis, so that values below the axis indicate inbreeding depression, and values above heterosis. Error bars correspond to the standard error.

Discussion

Effects of mating system

Cumulative inbreeding depression recorded for the three populations of *M. smejkalii* studied was comparable to values reported for typically outcrossing populations (0.44 and 0.58; Husband & Schemske 1996 and Winn et al. 2011, respectively), with little variation among populations and combinations of traits. This is consistent with the observations indicating a mixed-mating system in *M. smejkalii* (Stojanova et al. 2020). However, the timing of inbreeding depression (no significant early-acting and high late-acting inbreeding depression) is consistent with results reported for self-fertilizing species. In self-fertilizing species, early-acting, deleterious mutations are more effectively purged than in cross-fertilizing species (Husband & Schemske 1996). The unusual timing of inbreeding depression in *M. smejkalii* could be due to the joint effects of biparental inbreeding and genetic drift in fragmented populations, which increase the inbreeding coefficient of the offspring even when apparent outbreeding occurs (see below). Moreover, failure to detect statistical evidence for inbreeding depression does not necessarily indicate its absence. Thus, the possibility for early-acting inbreeding depression in *M. smejkalii* should not be completely overlooked solely on the basis of significance testing (Hedrick & Kalinowski 2000).

Cumulative estimates also showed that between-population outbreeding generally results in heterosis. However, cumulative estimates of heterosis ranged from weak outbreeding depression to high heterosis depending on the trait combination. Furthermore, when Z6 was used as the pollen donor, the between-population outbred offspring had lower heterosis than the offspring resulting from other pollen donors. Comparison of our results with those of other studies is difficult due to the lack of information on heterosis and outbreeding depression (Edmands 2007, Frankham 2015). However, since the effects of heterosis are mostly dependent on stochastic population differentiation created by genetic drift, one could expect more variation in the estimates of heterosis than in those of inbreeding depression (Escobar et al. 2008).

Variation in time

Inbreeding depression was higher in 2018 than in 2019 in all populations, and so was heterosis in population Z6. Variation in inbreeding depression among years is reported in other perennial herbaceous plants (Johnston 1992, Montalvo 1994, Sletvold et al. 2013, Sandner & Matthies 2016) and trees (Koelewijn & van Damme 2005, Nickolas et al. 2019). However, there is no general trend as observations differ among studies, populations in the same study and different traits within the same population.

There are several non-exclusive explanations for the annual variation in inbreeding depression in *M. smejkalii*. First, inbreeding and outbreeding depression may be environment-dependent and annual variation in the environment was not accounted for in this study. Second, selective mortality of inbred (and outbred) individuals could reduce inbreeding depression (and outbreeding depression) for traits other than survival. For instance, in two species of *Eucalyptus* (Nickolas et al. 2019), inbreeding depression of growth was reduced over the course of 23 years, while at the same time it increased for mortality. In line with this, mortality in *M. smejkalii* was higher in inbred relative to outbred offspring in Z1 and Z2. Similarly, in Z6 there was a higher mortality of offspring sired by Z2 (but not Z1), which could have decreased heterosis in 2019.

Finally, the changes in the magnitude of inbreeding and outbreeding depression could be due to differential gene expression between years induced by epigenetic modifications. For instance, in *Scabiosa columbaria* methylation levels are significantly lower in inbred than in outbred individuals, and global demethylation decreases inbreeding depression (Vergeer et al. 2012). To confirm this hypothesis, an examination of the genetic and epigenetic regulation of trait expression in ageing plants of *M. smejkalii* is required.

Variation in space: Effects of population

Populations Z1 and Z6 were small, so fewer plants were sampled, and they have a lower genetic diversity than population Z2. The putative absence of stage-specific inbreeding depression in Z6 and the low inbreeding depression in Z1 coupled with high heterosis in both populations are likely to be the consequence of genetic drift rather than purging of deleterious alleles. Both reduce within-population genetic variation: genetic drift through stochastic fixation and purging through selective elimination of deleterious alleles, effectively reducing the inbreeding depression estimates. Contrary to purging, the population mutation load does not decrease when accumulated through drift, thus decreasing the population mean performance relative to other populations (Hedrick & Kalinowski 2000, Barrett & Harder 2017). This is indeed the case with Z6 (mean length, number of stems and flower set were lower than in Z2 in 2018), but not with Z1 (Electronic Appendix 13). The effects of inbreeding are visible only as increased heterosis in between-population outbreed offspring, coming from the complementation of deleterious loci between differentiated populations, which is the case in both, Z1 and Z6. In population Z1, the benefits of heterosis could be lower because they are partly cancelled by outbreeding depression (Escobar et al. 2008). Inversely, because of its large size, it is likely that Z2 has endured less genetic drift, as indicated by its higher heterozygosity and lower F_{IS}. This can effectively result in higher inbreeding depression, and lower overall benefits of heterosis. Similar observations are reported for other endangered (Frankham 2015) and common species (e.g. Hedrick & Kalinowski 2000, Keller & Waller 2002, Luijten et al. 2002, Bailey & McCauley 2006, Escobar et al. 2008).

Intriguingly, *M. smejkalii* showed very little between-population genetic differentiation in the field, according to pairwise F_{ST} estimates from RADSeq SNPs (Z6-Z1: 0.038, Z6-Z2: 0.033, Z1-Z2: 0.039; Stojanova et al. 2020). This could be due to a genetic lag caused by the longevity of this species, which delays the consequences of genetic drift in recently disturbed natural populations of perennials, and makes it only detectable in their offspring, which are highly inbred (Schaal & Leverich 2004). Indeed, in Z6 the number of available parents for the controlled crosses was very low because of low germination and flowering, whereas in Z1 the parents were produced by seeds harvested from only seven individuals remaining in the natural population. Lastly, the non-uniform variation in inbreeding depression among populations can also be attributed to the stochastic effects of genetic drift, which result in a unique combination of fixed adaptive (and non-adaptive) genes for each population, with different consequences for the performance of inbred and outbred offspring (Bouzat 2010).

When averaged across all experimental environments, we only detected significant outbreeding depression in Z2 in 2018, suggesting that the benefit of reducing inbreeding depression compensates for the possible introduction of locally non-adapted genotypes in Z1 and Z6 (Fenster & Galloway 2000). This result was expected, as the populations included in this study fall in the category of low outbreeding risk according to Frankham et al. (2011): recently isolated and with similar (edaphic) environments. The benefits of heterosis are also reported in various other taxa of endangered (Frankham 2015), and non-endangered (e.g. Escobar et al. 2008, Wagenius et al. 2010) species. There is, however an environment-dependent effect on heterosis that may warrant some precautions regarding conservation.

Variation in space: Effects of the environment

We recorded a decrease in inbreeding depression in stressful environments, which seems to contradict the general view that environmental stress increases inbreeding depression (Armbruster & Reed 2005, Fox & Reed 2011). A growing number of studies nevertheless report decreases in inbreeding depression in stressful environments (Waller et al. 2008, Sandner & Matthies 2016). According to Waller et al. (2008), the amount of phenotypic variation is a better predictor of the magnitude of inbreeding depression than level of environmental stress, with inbreeding depression greatest in environments in which there is the highest phenotypic variation (i.e., opportunity for selection against inbred offspring). The positive, qualitative association we detected between inbreeding depression and phenotypic variation for all late traits of *M. smejkalii* is in line with this hypothesis.

Heterosis also increased with increase in environmental stress based on the positive quantitative association we detected between heterosis and environmental stress. Enhancement of heterosis in stressful environments is reported in other species, especially cultivated crops (Prill et al. 2014 and references therein), but also in endangered species (Frankham 2015) and can offer some interesting perspectives on species conservation as discussed below.

Conclusions and conservation recommendations

Altogether, the results of this study offer a positive prospective for the conservation of *M. smejkalii*. First, the negative effects of inbreeding present in all the populations studied can be counteracted by heterosis, especially in the smallest, most vulnerable populations. Second, inbreeding depression generally decreases in the stressful, serpentine habitats. Third, we detected no early-acting inbreeding depression in the greenhouse. Fourth, late-acting inbreeding and outbreeding depression decreased in the second year. Lastly, serpentine soil seems to negatively affect germination regardless of the occurrence of inbreeding/outbreeding depression in *M. smejkalii,* which may limit the efficiency of rescuing populations by introducing seed. Based on these results, conservation is best achieved by introducing between-population hybrid offspring that were previously grown in a controlled environment. Although generally more time consuming than introducing seed, this approach avoids the delicate stage of seedling recruitment, which can be particularly difficult for *M. smejkalii*. Moreover, introducing plants rather than sowing seed protects the populations from the consequences of outbreeding depression that occurred during the first year in some of the populations. If, however, population restoration is carried out by sowing seed, we recommend using seed from the same population, along with careful monitoring of the new juveniles in each season.

See www.preslia.cz for Electronic Appendices 1–14.

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Souhrn

Heterózní efekt může být využit při pokusech o záchranu ohrožených populací rostlin. Máme však málo znalostí o tom, jak inbreeding, heterózní efekt a outbrední deprese společně ovlivňují zdatnost druhů s malými, fragmentovanými populacemi. Pochopení společného působení těchto procesů je dále komplikováno jejich mezipopulační variabilitou, variabilitou prostředí v čase a stochastickými vlivy genetického driftu. Pro pochopení těchto procesů jsme srovnávali po dobu dvou let růst potomků ručně opylovaných rostlin ze tří přirozených populací *Minuartia smejkalii* ve dvou typech půdy a třech konkurenčních prostředích. U časných vývojových fází ve skleníku jsme nezjistili žádnou inbrední nebo outbrední depresi. U pozdějších růstových fází v zahradě byla u menších populací inbrední deprese nízká a mohla být potlačena heterózním efektem. Jak inbrední deprese, tak heterózní efekt se lišily mezi hodnocenými parametry, jednotlivými roky i prostředím. Se zvyšujícím se stresem prostředí a klesající fenotypovou variabilitou míra inbrední deprese klesala. Heterózní efekt se zvyšoval s rostoucím stresem prostředí. Výsledky ukazují, že do malých populací *M. smejkalii* je vhodné pro posílení heterózního efektu vnést mezipopulační křížence. Přenos poznatků na jiné druhy či dokonce na jiné populace u kuřičky hadcové je nutné provádět s opatrností zejména kvůli stochastickým vlivům genetického driftu, které se mohou mít jedinečný dopad v každé populaci.

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