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Early-acting inbreeding depression in a clonal dwarf shrub, *Vaccinium myrtillus*, in a northern boreal forest

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Seed yield reductions upon self-pollination in comparison to cross-pollination can be caused by either self-incompatibility or inbreeding depression. Several ericaceous species have shown partial self-sterility, most probably due to inbreeding depression expressed at seed maturation. We conducted pollination experiments in *Vaccinium myrtillus* (the bilberry) in a northern boreal forest and used the results on seed production in a regression model to examine the occurrence of inbreeding depression *vs*. prezygotic self-incompatibility. We also estimated the magnitude of inbreeding depression and the number of lethal equivalents per zygote for bilberry. We estimated an inbreeding depression of $\delta = 0.8$ and a mean of 7.8 lethal equivalents per zygote (range of 1–14). These figures are comparable to the estimates made for bilberry at a location in central Europe and for other long-lived plants, and support the observation that longevity is associated with high genetic load.

Key words: clonal growth, cross-pollination, Ericaceae, inbreeding depression, lethal equivalents, self-pollination, *Vaccinium myrtillus*

Introduction

Plant reproductive success, especially seed set, may be reduced following self-pollination in comparison to cross-pollination because a species is self-incompatible or suffers from inbreeding depression. Self-incompatibility is defined as "the partial or complete inability of a functionally bisexual seed plant to produce zygotes after self-pollination" (Becerra & Lloyd 1992: p. 465). In the strict sense, self-incompatibility mechanisms function before fertilization takes place, but in some plant species expression may occur after fertilization (late-acting self-incompatibility; Seavey & Bawa 1986). Also, it has been found that the expression of self-incompatibility varies in some species with environmental conditions (Becerra & Lloyd 1992), resource availability (Reinartz & Les 1994), genetic differences between individuals (Crowe 1971, Reinartz & Les 1994, Vogler *et al*. 1998), or flower age (Shivanna & Rangaswamy 1969, Levin 1984, Vogler *et al*. 1998, Goodwillie *et al*. 2004). Self-incompatibility may not be expressed in very young flowers, and bud pollination has been utilized to produce self-seeds in incompatible plants (*see* e.g. Levin 1993, Kärkkäinen *et al*. 1999). On the other hand, self-incompatibility

may also lose its strength with increasing flower age (Vogler *et al*. 1998).

Inbreeding depression, the reduction in fitness of offspring from related parents compared with offspring from unrelated parents, can be expressed at different stages of a plant's life cycle (e.g. embryo development, growth and survival of progeny or reproduction), and may be especially strong during seed development, a time when numerous genes are expressed (Stevens & Bougourd 1988, Husband & Schemske 1995, Kennington & James 1997). Due to a higher mutation rate per generation, long-lived species seem to have in general higher genetic loads (Wiens 1984, Klekowski 1988, Klekowski & Godfrey 1989). Also, there appears to be a relation between plant growth form and the predominant mode of fertilization. Barrett *et al*. (1996) reported that the frequency of self-fertilization is lower in woody perennial species than in herbaceous perennials or annuals. Theoretical studies have anticipated that repeatedly self-fertilizing populations or species exhibit less inbreeding depression than cross-fertilizing ones because the deleterious alleles, especially those with major effects, will be gradually removed from the genome (Lande & Schemske 1985, Charlesworth & Charlesworth 1987, Husband & Schemske 1996, Crnokrak & Barrett 2002).

The heath family, Ericaceae, contains about 1800 species in 70 genera. Plants are dwarf shrubs or small trees with belowground rhizomatous growth. Flowers are generally hermaphrodite, and anemophilous or entomophilous. Ericaceae are represented in the tropics, in the temperate zones and in the Arctic (Gleason 1958), and as clonal dwarf shrubs they dominate the field layer in boreal forests. Because of their clonal growth form, Ericaceae can be expected to be comparable to long-lived trees with a high genetic load and hence inbreeding depression. Reduced self-fertility has been reported for a number of ericaceous species such as *Kalmia latifolia* (Rathcke & Real 1993), *Calluna vulgaris* (Mahy & Jacquemart 1999), several species within the genus *Vaccinium* (Krebs & Hancock 1990, 1991, Guillaume & Jacquemart 1999, Hokanson & Hancock 2000, Raspé *et al*. 2004), and has been explained to be a result of early-acting inbreeding depression. High inbreeding depression during seed maturation (δ = 0.837) was calculated for *Vaccinium myrtillus* in a heathland population in the Upper Ardennes, Belgium (Guillaume & Jacquemart 1999).

In the present work, we investigated the level of early-acting inbreeding depression of *Vaccinium myrtillus* at seed maturation in a population in a northern boreal forest. First, we conducted hand-pollinations at different flower ages with cross- and self-pollen to investigate the longevity of stigma receptivity, and to determine reproductive success. Although reduced self-fertility in bilberry has been attributed to inbreeding depression elsewhere, we decided to rule out possible temporal variation in reproductive success following selfing based on the knowledge that the expression of self-incompatibility may be variable. Using the results from the hand-pollinations, we then introduce a regression method to evaluate the presence of processes affecting seed maturation prior to or after fertilization (self-incompatibility versus early-acting inbreeding depression). We also estimated the magnitude of inbreeding depression and calculated the number of lethal equivalents from the relative survivorship of selfed to crossed progeny at the seed stage following the method of Morton *et al*. (1956) and Sorensen (1969).

Material and methods

Study species and sites

Vaccinium myrtillus is a deciduous clonal dwarf shrub. Aerial shoots (ramets) are about 30 cm in height and produce reduced inflorescences with singular flowers or flower pairs. The flowers produce nectar and are slightly protandrous (Hegi 1927). Pollen is dispersed in tetrads, and anthers open with small pores. Several flowers are formed by a ramet, and they may be open at the same time.

We performed pollination experiments with bilberry at Oulanka National Park, near Kuusamo (66°20´N, 29°20´E) in northern Finland at a natural site in a spruce-dominated mesic heath forest in 1994 and 1995, and in an experimental field in 1996. The experimental field was set up in the close vicinity of the Oulanka Biological Station. The soil consisted of a mixture of peat and sand (3:1). In autumn 1993, we dug up parts of

bilberry genets from a spruce-dominated forest approximately three kilometres from the Oulanka Biological Station. The spatial dimensions of bilberry clones in this forest are not known. According to Flower-Ellis (1971), in Swedish forest sites bilberry clones are up to 5.5 m in length. We tried to avoid sampling the same bilberry genets by leaving ca. five metres distance between the excavation points. The samples consisted of a rhizome fragment and at least one, but often several ramets. We planted a total of 76 fragments, each to an individual square in the experimental field. The squares were 1 m × 0.9 m and bordered by fabric to a soil depth of ca. 60 cm to keep neighbouring bilberries separate. After planting, new ramets were produced by the fragments, and we will call the entity of all ramets within a square a 'clone'.

Hand pollinations

Prior to the hand pollinations, we caged the experimental ramets to exclude pollinators, marked the flower buds and inspected them daily to assess flower age. The cages consisted of a frame made of metal wire that was covered with nylon netting. For all hand pollinations, we shook pollen from donor flower(s) onto a glass microscope slide and transferred the pollen to the recipient flower by touching the stigma with the slide (as described in Molau *et al*. 1989). To produce pollen mixtures, we spread self- and cross-pollen one after the other as evenly as possible onto the slide, and transferred pollen from the different places across the slide to the stigma. We checked with a hand-lens to ensure that the stigmas were covered with pollen, but we have no estimations on the qualitative nor quantitative composition of the mixture thus applied. The average bilberry flower had 83 ± 2 ovules per flower (n = 30) at the forest site in 1994, and 77 ± 2.3 ovules per flower (n = 43) in 1995 (counted from flowers preserved in 70% ethanol).

Experiment I: Cross-pollinations at different flower ages in the natural population

We performed hand-cross-pollinations to test the duration of flower stigma receptivity. This knowledge is important to gain before conducting pollen chase experiments (compare Exp. II and Exp. III) such that the chase pollen is applied onto a receptive stigma. In 1994, we chose a total of 56 bilberry ramets that were grouped in eight plots each with seven bilberry ramets at the natural forest site. The ramets within each group (= plot) were separated by ca. 1–5 metres, and the plots were distanced by at least 10 metres from the next adjacent plot. We cross-pollinated one flower per caged ramet with pollen from two different bilberry flowers from within the same population. The pollen donor ramets were ca. ten metres apart from the pollen recipient. The flower age at the time of pollination was either 1 day, 2 days, 3 days, 4 days, 5 days, 7 days, or 10 days. Each pollination treatment was performed in each of the eight replicate plots.

Experiment II: Self-pollinations and pollen chase experiment in the natural population

In 1995, we marked a total of 90 bilberry ramets (ten plots with nine ramets each) at the natural forest site and applied nine pollination treatments (no hand pollination, five different self-pollination and three pollen chase treatments) randomly to one ramet each per plot. Thus, each pollination treatment was performed on ten replicate ramets. All ramets were caged, and the distance between the plots and ramets was as described previously. Within a plot we applied the following treatments. To test whether apomixis could occur, we emasculated all flower buds on one ramet and left them unpollinated. To investigate for possible temporal differences in the ability to set fruit and seed upon self-pollination, we self-pollinated different flowers at either 0 (bud pollination), 2, 4, 6 and 8 days of age. Each pollination treatment was applied to a different ramet. We chose two flowers per ramet as pollen recipients and transferred pollen from another flower within the same ramet. For the bud pollinations, flowers were emasculated and pollinated when still in the bud stage. For the other self-pollinations, we emasculated flowers immediately after opening and pollinated them when the recipient flower had reached the appro-

priate treatment age. Thereafter, we emasculated all remaining flowers on the ramet to avoid any possible later occurring within-ramet self-pollination (geitonogamy).

We subjected three caged ramets in each plot to a combination of self- and cross-pollinations without previous emasculation, to test whether successive or simultaneous self- and cross-pollination affected reproductive success. We applied the following treatments: self-pollination at flower age 3 d followed by cross-pollination after either one or three days (pollen chase), or application of a mixture of self- and cross-pollen onto 3-days old flowers (simultaneous self- + cross-pollination). We chose the time delay of one or three days between self- and cross-pollination to ensure that the self-pollen had enough time to grow through the style and reach the ovules. We collected cross pollen from the same bilberry population from two flowers of different ramets that were several metres away from the experimental ramet.

Experiment III: Self-pollinations and pollen chase experiment in the experimental field

In order to calculate inbreeding depression at seed maturation, we performed self-pollination and cross-pollination in the same clone. Also, conducting the pollinations in the experimental field attempts to minimize small-scale environmental variation that is likely to be encountered within the forest habitat. In 1996, we chose one ramet each from sixteen bilberry clones and caged a total of 33 flowers (mean 2.1 flowers/ramet, range 1–6). We left the flowers unpollinated to assess whether bilberry is capable of autogamy (autonomous self-fertilization). Furthermore, we chose fifteen clones and caged two ramets of each clone before the flower buds opened. One flower on each ramet was randomly assigned to one of the following five treatments: (i) self-pollination; self- followed by cross-pollination after either (ii) 1 d or (iii) 2 d (pollen chase); (iv) pollination with a mixture of self- and cross-pollen (simultaneous self- + cross-pollination). We conducted self-pollinations between flowers within the same ramet, and (v) cross-pollinated one flower on the other caged ramet with pollen from a different bilberry clone. Thus, each clone received five pollination treatments.

Experiment IV: Self- *vs.* cross-pollinations in the experimental field

Competition between fruits is possible when several berries are maturing on the same ramet at the same time. We conducted an experiment where only two flowers per ramet were pollinated. We chose eleven clones from the experimental field and caged two ramets on each. We self-pollinated one flower of one caged ramet with pollen from a flower of the other caged ramet within the same clone, and cross-pollinated one flower with pollen from a different clone.

Fruit set and seed number

We estimated fruit set per treatment as the percentage of pollinated flowers developing into a berry. For each treatment, the data on all pollinated flowers across all ramets were used in this calculation. We collected the berries when they were ripe, removed mature seeds, aborted seeds and unfertilized ovules manually from the berries and counted them under a stereomicroscope against millimetre paper. We considered seeds mature if they were about 1 mm long and full. Unfertilized ovules were ca. one third of a millimetre long and flat, whereas aborted seeds were about 0.3–1 mm long, slightly swollen or clearly enlarged but flattened. When calculating treatment means for number of seeds, we considered the ramet as the unit of replication. Therefore, in Experiment II we calculated first the mean number of seeds per berry for each ramet, and then computed the treatment mean from the ramet means. In the same way, we estimated the mean number of aborted seeds and unfertilized ovules. In the other experiments, we applied each pollination treatment only once to each clone, and therefore the treatment mean was directly calculated across replicate clones. Note that instead of percentage seed set [(mature seeds/mature seeds plus aborted seeds plus unfertilized ovules) × 100] we used number of mature

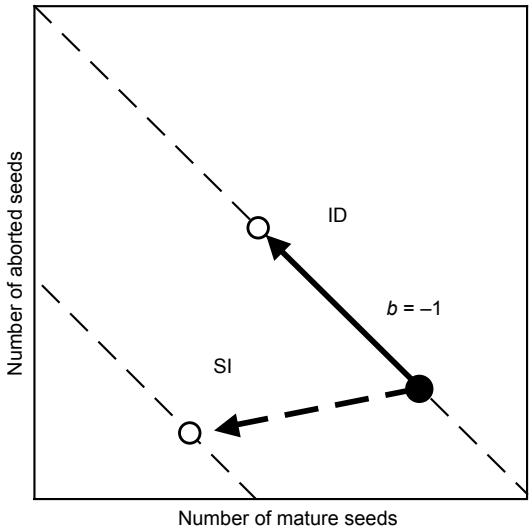

Fig. 1. Schematic representation on effects of inbreeding depression (ID) *vs.* self-incompatibility (SI). Hypothetical ratio of aborted to mature seeds in cross-pollinated fruits (●). In self-pollinated fruits (○), the ratio of aborted to mature seeds is expected to change with inbreeding depression in the direction of the solid arrow (slope of the line $b = -1$), but with self-incompatibility along the dashed arrow (slope of the line $b > -1$).

seeds as measure of reproductive success, and in the statistical tests, we used the total number of ovules as covariate.

Regression model, inbreeding depression and lethal equivalents

In order to give additional support to the assumption that early-acting inbreeding depression affects seed maturation in bilberry, we examined our seed data from self- and cross-pollinations with a regression model comparing the numbers of mature and aborted seeds per fruit (*see* Fig. 1). We assumed that the sum of mature seeds plus aborted seeds will remain constant following self- and cross-pollinations if inbreeding depression is present, i.e. the slope of the regression line of aborted seeds on mature seeds should be –1. On the other hand, if self-incompatibility prevents fertilization, the sum of seeds plus aborted seeds in self-pollinated fruits should be lower than in cross-pollinated ones, and the slope should significantly deviate from –1, being either less negative or positive. This regression method provides a way of comparing the outcome of self- and cross-pollination treatments that have been replicated in time or space. We discuss limitations of this model later on.

For those bilberry clones in the experimental field that yielded seeds in both self- and cross-pollinations, we quantified inbreeding depression and calculated the number of lethal equivalents expressed during the embryonic stage. Inbreeding depression was calculated as:

$$\delta = 1 - W_s/W_o, \quad (1)$$

where W_s = fitness of selfed progeny and W_o = fitness of crossed progeny (Frankham *et al.* 2002). The ratio W_s/W_o equals R, the relative survivorship of selfed progeny. Frankham *et al.* (2002: p. 301) defines a lethal equivalent as "a group of detrimental alleles that would cause on average one death if homozygous, e.g. one lethal allele, or two alleles each with 50% probability of causing death, etc." We calculated the number of lethal equivalents for bilberry, following Morton *et al.* (1956) and Sorensen (1969) using the equation:

$$2B = -4\ln R \quad (2)$$

where R is W_s/W_o and $2B$ is the average number of lethal equivalents per zygote.

Data analyses

We tested the differences in fruit set between the pollination treatments in Experiments II and III using the χ^2-test (Sokal & Rohlf 1995) based on frequencies of successful and unsuccessful pollinations (berry matured, berry not matured). When significant differences were found, we made pairwise comparisons employing Fisher's Exact Test (2×2) (Sokal & Rohlf 1995: p. 730). To account for multiple comparisons, we corrected the significance levels using the Dunn-Šidák method (Sokal & Rohlf 1995: p. 241). We also used Fisher's Exact Test to compare fruit sets in Experiment IV. Fruit set in Experiment I could not be tested statistically because the assumptions of the χ^2-test for expected frequencies were not met.

We investigated the differences among pollination treatments (fixed factor) in seed number

per fruit, number of aborted seeds per fruit, and number of unfertilized ovules per fruit separately by using ANCOVA. Blocks (plots or clones) were included as a random factor. To control the possible variation in ovule number between blocks and treated ramets, we used the total number of ovules as a covariate. Differences in the total number of ovules (sum of mature seeds, aborted seeds and unfertilized ovules) per fruit were examined using ANOVA (randomized complete block design) with pollination treatment as a fixed factor and plots (or clones) as random blocks. We made Šidák's post hoc multiple comparisons on the differences between treatments when more than two groups were tested (with Šidák's adjustment for multiple comparisons).

To meet the assumptions of normality and homoscedasticity, some of the data were transformed prior to analysis. The number of aborted seeds, unfertilized ovules and total ovules in Experiment I were $\log(x + 1)$ transformed. Seed number in Experiment II and Experiment IV was square-root $(x + 0.5)$ transformed. The number of aborted seeds and unfertilized ovules in Experiment III and the number of unfertilized ovules and total ovules in Experiment II were rank-transformed prior to analysis (e.g. Montgomery 1984). We performed the statistical tests with the SPSS (version 10.1, SPSS Inc., Chicago, Ill.) computer software program.

We computed the regression coefficient b (± SE) and tested whether it was significantly different from –1 (hypothesis H_0: $\beta = \beta_0$ and H_A: $\beta \neq \beta_0$, t-test). We calculated the 95% confidence interval for the regression coefficient with $L_1 = b - t_{0.05[8]}s_b$ and $L_2 = b + t_{0.05[8]}s_b$ (Sokal & Rohlf 1995: pp. 470–472).

Results

The fruit set differed in response to the pollination treatments. Flowers set few fruits when unpollinated, but both hand-self- and hand-cross-pollinations yielded many fruits with the exception of the two treatments when flowers were older (self at flower age 8 d, and cross at flower age 10 d). Seed numbers did not differ in cross-pollinated flowers of different age, or self-pollinated flowers of different age. Pure cross-pollinations yielded about five times more seeds than pure self-pollinations. Also pollen mixtures consisting of self- and cross-pollen yielded more seeds than pure self-pollinations. However, flowers matured as many seeds in self-pollination followed by cross-pollination after 1 d, 2 d, or 3 d as in pure self-pollinations.

Experiment I: Cross-pollinations at different flower ages in the natural population

Fruit set following hand cross-pollinations was between 75% and 88% at flower ages of 1 d to 4 d, 50% at 7 d of age and 0% at 10 d of age (individual fruit set data not shown). Hand cross-pollinations at different flower ages (1 d, 2 d, 3 d, 4 d, 5 d and 7 d) had no significant effect on mean number of mature seeds per berry ($F_{5,21} = 1.423$, $P = 0.257$), mean number of aborted seeds per berry ($F_{5,21} = 2.136$, $P = 0.101$), mean number of unfertilized ovules ($F_{5,21} = 0.198$, $P = 0.960$), or mean number of total ovules ($F_{5,22} = 0.206$, $P = 0.956$). The covariate (number of ovules) had a significant effect on mean number of mature seeds ($F_{1,21} = 14.021$, $P = 0.001$) and on number of aborted seeds ($F_{1,21} = 4.595$, $P = 0.044$). Also, blocks differed significantly for the number of aborted seeds per berry ($F_{7,21} = 2.747$, $P = 0.034$), and the total number of ovules ($F_{7,22} = 3.976$, $P = 0.006$). The individual seed data are not shown; instead, we present the mean value from the pollination treatments cross at 1 d, 2 d, 3 d, 4 d, and 5 d in Fig. 2.

Experiment II: Self-pollinations and pollen chase experiment in the natural population

Bilberry was not apomictic, as none of the emasculated caged flowers set fruit (Table 1, unpollinated). Self-pollination yielded high fruit sets (80%–85%) at flower ages of 0 d, 2 d and 4 d (Table 1). At 6 days of age, fruit set was still more than 50%, but was 0% at 8 d of age. Fruit set was also high (85%–90%) when self-pollination was followed by cross-pollination, and 100% when self- and cross-pollen were applied

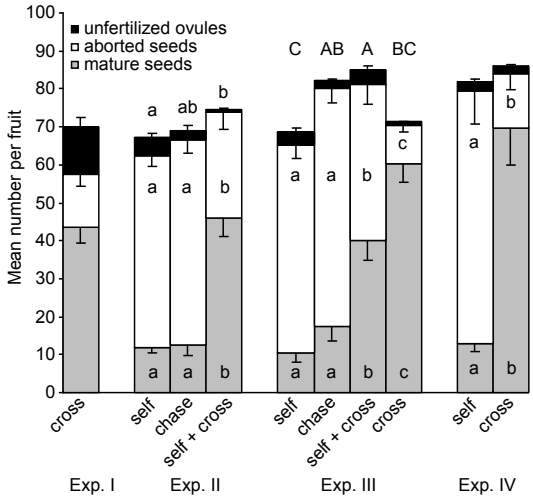

Fig. 2. Mean number (and SE) of mature seeds, aborted seeds and unfertilized ovules in *Vaccinium myrtillus* in the pollination treatments: self-pollination, self- followed by cross-pollination (chase), simultaneous application of self- and cross-pollen (self + cross), and cross-pollination at the forest site (Experiments I and II) and in the experimental field (Experiments III and IV). Different letters indicate significant differences in pollination treatments following pairwise comparisons ($P < 0.05$) after two-way ANOVA. For the respective experiments, different small letters above the columns denote significant differences between the treatment means for the number of unfertilized ovules, and different small letters within the columns for the number of aborted seeds and the number of mature seeds. Different capital letters above the columns in Exp. III denote differences in the total number of ovules. Absence of letters indicates that there were no significant differences between treatments in a given experiment. Sample sizes were 8–15 ramets per treatment.

at the same time (Table 1). When all the pollination treatments were pooled together, pollination had a significant effect on fruit set ($\chi^2 = 124.43$, df = 8, $P < 0.001$). Pairwise tests showed that the treatments unpollinated, and self-pollinated at flower age 8 d both differed significantly from each of the fruit sets of the other hand-pollination treatments.

We investigated the seed data in two steps. First, there was no difference in seed number between self-pollination treatments at 0 d, 2 d, 4 d and 6 d (ANCOVA: pollination, $F_{3,17} = 2.552$, $P = 0.090$; block, $F_{9,17} = 2.041$, $P = 0.098$; covariate, $F_{1,17} = 1.823$, $P = 0.195$), and neither between the two pollen chase treatments self- followed by cross-pollen after 1 d and 3 d (ANCOVA: pollination, $F_{1,7} = 2.218$, $P = 0.180$; block, $F_{8,7} = 1.730$, $P = 0.242$; covariate, $F_{1,7} = 0.009$, $P = 0.925$). We therefore calculated a mean value for seed number per fruit within each block from the self-pollination treatments (at 0 d, 2 d, 4 d and 6 d), and a separate mean from the pollen chase treatments (self- followed by cross-pollen after 1 d and 3 d).

Second, the treatments that we investigated in the further test were self-pollination, pollen chase (self- followed by cross-pollen after 1 d and 3 d), and simultaneous self- + cross-pollination (application of a pollen mixture of self- and cross-pollen). Pollination treatment ($F_{2,16} = 32.568$, $P < 0.001$; Fig. 2) and the covariate ($F_{1,16} = 5.542$, $P = 0.032$) had a significant effect on mean seed number per fruit. Flowers that received the pollen chase treatment matured as many seeds as flowers in self-pollination only (Fig. 2). However, when cross-pollen was applied simultaneously with self-pollen, the number of seeds per fruit was greater than in the pollen chase treatment. The pollination treatment ($F_{2,16} = 19.998$, $P < 0.001$) and the covariate ($F_{1,16} = 5.488$, $P = 0.032$) had both a significant effect on mean number of aborted seeds (Fig. 2). Abortion was greater in the self-pollination only treatment than in the simultaneous self- + cross-pollination. The pollination treatment affected the number of unfertilized ovules per fruit significantly ($F_{2,16} = 10.679$, $P = 0.001$), such that fruits from self-pollinations had more unfertilized ovules than fruits from the simultaneous self- + cross-pollination treatment. However, the variation is small in relation to the number of aborted seeds.

Experiment III: Self-pollinations and pollen chase experiment in the experimental field

Autonomous self-fertilization may be possible, although only one out of 33 caged unpollinated flowers in the experimental field produced a berry (Table 1). It is possible that this fruit was a result of contamination. Fruit set in the hand-pollination treatments ranged from 70% to 100% (Table 1), and a significant difference was detected ($\chi^2 = 72.745$, df = 5, $P < 0.001$). The fruit set in the unpollinated treatment (Table 1)

differed significantly from the hand-pollination treatments in pairwise tests.

We calculated the mean from the two pollen chase treatments (self- followed by cross-pollen after 1 d and 2 d), because they did not differ from each other (ANCOVA: pollination, $F_{1,5}$ = 0.738, P = 0.430; block, $F_{14,5}$ = 0.682, P = 0.738; covariate, $F_{1,5}$ = 5.115, P = 0.073).

We then tested the treatments self-pollination, pollen chase, simultaneous self- + cross-pollination, and cross-pollination in further ANCOVA. The mean number of mature seeds was significantly affected by pollination treatment ($F_{3,36}$ = 45.388, P < 0.001) (Fig. 2), by block ($F_{14,36}$ = 1.997, P = 0.048) and the covariate ($F_{1,36}$ = 18.104, P < 0.001). Like in Experiment II, the pollen chase treatment yielded as many mature seed per fruit as did self-pollination only, and simultaneous self- and cross-pollination yielded more seeds than the pollen chase treatment (Fig. 2). Cross-pollinated flowers matured more seeds than flowers that were self-pollinated only, or that were selfed and crossed simultaneously (Fig. 2). The pollination treatment ($F_{3,36}$ = 33.562, P < 0.001) and block ($F_{14,36}$ = 2.402, P = 0.017) had a significant effect on mean number of aborted seeds. Abortion was greater in the self-pollination only treatment than in the simultaneous self- + cross-pollination and the cross-pollination only treatments. The total number of ovules differed among pollination treatments ($F_{3,37}$ = 7.822, P < 0.001) and blocks ($F_{14,37}$ = 3.553, P = 0.001).

Experiment IV: Self- *vs.* cross-pollinations in the experimental field

The fruit sets following self-pollinations and cross-pollinations did not differ from each other (Fisher's Exact Test: P = 0.214). Cross-pollinated flowers matured more seeds ($F_{1,6}$ = 53.663, P < 0.001) and aborted fewer seeds ($F_{1,6}$ = 27.042, P < 0.002) than those that were self-pollinated only (Fig. 2). The total number of ovules differed among blocks ($F_{7,7}$ = 5.982, P = 0.015).

Effect of emasculation

Emasculation did not have any detrimental effect on berry or seed production. Emasculated flowers that were hand self-pollinated (Experiment II) produced similar numbers of fruits as hand cross- or hand self-pollinated flowers that had not been emasculated (Experiments I, III and IV). Also, when the seed number per berry in emasculated self-pollinated flowers (mean ± SE = 11.8 ± 1.4, Experiment II) was compared with

Table 1. Fruit set (%) in *Vaccinium myrtillus* in response to different pollination treatments at the forest site (Experiment II) and on the experimental field (Experiments III and IV).

Pollination treatment	Exp. II		Exp. III		Exp. IV	
	%	*n*	%	*n*	%	*n*
Unpollinated*	0.0	52	3.0	33		
Self in bud-stage	85.0	20				
Self at flower age 2 d	81.3	16				
Self at flower age 4 d	80.0	15				
Self at flower age 6 d	56.3	16				
Self at flower age 8 d	0.0	15				
Self, and cross after > 1 d†	89.5	19	69.2	13		
Self, and cross after 1 d	86.7	15	86.7	15		
Self and cross at same time	100.0	16	100.0	15		
Self (flower age 1–4 d)‡			85.7	14	72.7	11
Cross			100.0	13	100.0	11

Note: All ramets were caged prior to pollination to exclude natural pollinators. *n* = number of flowers, equals number of clones in Experiments III and IV. *Flowers were emasculated at the forest site, testing for agamospermy. Fruit set in unpollinated flowers in the experimental field tested for autogamy. †Cross-pollination followed self-pollination at the forest site after 3 days, and after 2 days in the experimental field. ‡Self-pollination was within the same ramet in Experiment III, and between sister ramets of the same clone in Experiment IV.

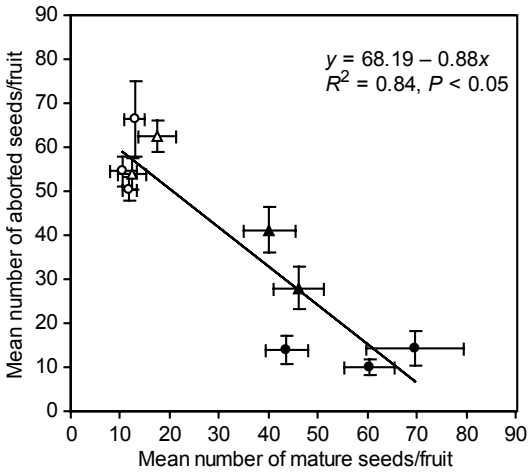

Fig. 3. Mean number of aborted seeds (± SE) plotted against the mean number of mature seeds per fruit (± SE) as yielded in the pollination experiments with *Vaccinium myrtillus* in the forest (Experiments I and II) and in the experimental field (Experiments III and IV). The pollination treatments were: self-pollination (○), self- followed by cross-pollination (△), simultaneous application of self- and cross-pollen (▲), and cross-pollination (●).

Table 2. Seed numbers matured per fruit and number of aborted seeds per fruit in self-pollinations (self) and cross-pollinations (cross), relative survivorship (R) of offspring from self-pollination/offspring from cross-pollination, inbreeding depression (δ) and number of lethal equivalents ($2B$) calculated for *Vaccinium myrtillus* in the experimental field.

Clone number	# Mature seeds		# Aborted seeds				
	self	cross	self	cross	R	δ	$2B$
1	1	32	56	14	0.03	0.97	14.03
2	3	67	57	18	0.04	0.96	12.88
3	5	78	101	11	0.06	0.94	11.25
4	4	52	62	8	0.08	0.92	10.10
5	8	85	41	4	0.09	0.91	9.63
6	7	76	68	5	0.09	0.91	9.63
7	5	55	63	31	0.09	0.91	9.63
8	8	82	48	7	0.10	0.90	9.21
9	7	55	73	20	0.13	0.87	8.16
10	19	120	85	4	0.16	0.84	7.33
11	17	98	82	8	0.17	0.83	7.09
12	13	75	55	3	0.17	0.83	7.09
13	15	59	77	19	0.25	0.75	5.55
14	19	68	60	3	0.28	0.72	5.09
15	18	56	43	21	0.32	0.68	4.56
16	14	41	28	30	0.34	0.66	4.32
17	17	42	40	7	0.40	0.60	3.67
18	35	47	35	5	0.74	0.26	1.20

the seed number in flowers that were left intact (10.6 ± 2.7, and 12.9 ± 2.2, in Experiment III and IV, respectively), there was no obvious difference. The fact that the self-pollination treatment on emasculated flowers at an age of 8 d did not yield any seeds, indicates that no unintentional pollination and fertilization took place while emasculating the flowers.

Regression model, early-acting inbreeding depression and lethal equivalents

When we applied the regression method as displayed in Fig. 1 to our results (Experiments I–IV), the slope of the regression line calculated for the different pollination treatments was –0.88 (± SE = 0.137, confidence interval L_1 = –1.197 and L_2 = –0.565) (Fig. 3). The regression coefficient did not significantly deviate from –1 (t = 0.8759, df = 8, $P > 0.05$). Consequently, the seed yield of self-pollinated bilberry plants is reduced because of post- rather than pre-fertilization processes. This assumes that variation in the total number of ovules does not confound the results. Since there was some variation in total ovules (*see* Experiment III above), we also calculated the regression by using abortion rate [number of aborted seeds/(number of aborted seeds + mature seeds)] and seed set [number of mature seeds/total ovules]. The data gave the same graph as in Fig. 3, with a slightly steeper slope b = –1.05 (± SE = 0.052).

We calculated that the *Vaccinium myrtillus* plants in the experimental field showed a mean (± SE) inbreeding depression at the embryonic stage of 0.8 ± 0.04 and harboured a mean (± SE) number of lethal equivalents per zygote of 7.8 ± 0.8 (n = 18) (range 1 to 14 lethal equivalents; Table 2). Low relative zygote survivorship (R < 0.2) occurred in twelve out of eighteen clones. Seventeen out of the eighteen clones had R < 0.5.

Discussion

Flower age did not seem to affect seed number following either self- or cross-pollination, as

long as flowers were still functional. In some plant species, seed set following selfing has been reported to increase with ageing of the flowers. In *Campanula rapunculoides*, this has been attributed to an age-dependent breakdown of the self-incompatibility system (Vogler *et al*. 1998). Goodwillie *et al*. (2004) detected a transient form of self-incompatibility in *Leptosiphon jepsonii*, where pollen tube growth was reduced in self-pollinated one-day-old flowers as compared to cross-pollinated flowers of the same age or self-pollinated flowers aged two to three days. The ability of bilberry flowers to set fruit decreased from the age of 6 d onwards, presumably because the stigmas ceased to be receptive. In *Vaccinium angustifolium*, flowers were capable of setting fruit under natural pollination conditions up to 7 d of age (Wood 1962). In our pollination experiments with bilberry, fruits and seeds were matured in both cross- and self-pollinated plants, but seed number was much lower following selfing than following crossing. If bilberry was fully self-incompatible, no fruits would have been expected to mature following selfing, but instead there was no difference in fruit set between self- and cross-pollinations.

In the pollen chase experiments, when cross-pollen was applied one day or more after self-pollen, the number of seeds per fruit was as high as that following self-pollination only. Also, the number of aborted seeds in self-pollination only and pollen chase treatments equalled each other and was significantly greater than after the simultaneous application of self- and cross-pollen or cross-pollen only. Most of the ovules had probably been fertilized by self-pollen and some were aborted. Therefore, the later arriving cross-pollen was not able to fertilize any more ovules. Krebs and Hancock (1990) reported similar results in pollen-chase experiments with *Vaccinium corymbosum*, only on a different time scale: cross-pollen when applied one day after self-pollen yielded as many seeds as did pure cross-pollination, but when cross-pollen was applied 2 d to 4 d after self-pollen, seed set did not differ from that following self-pollinations. When we applied self- and cross-pollen simultaneously to bilberry flowers, the seed number per fruit was greater than that following selfing only, but was smaller than that following cross-ing only. The same has been observed in *Vaccinium corymbosum* (Vander Kloet & Lyrene 1987), *Calluna vulgaris* (Mahy & Jacquemart 1999) and *Vaccinium myrtillus* in central Europe (Raspé *et al*. 2004) where seed set following application of a mixture of self- and cross-pollen ranged between seed set following self- or cross-pollination.

However, results from pollen-chase experiments are not entirely unambiguous. In some self-incompatible species it has been observed that self-pollen can negatively affect cross-pollen although self-pollen did not fertilize ovules. Self-pollen has been reported to reduce pollen tube growth of cross-pollen (Ockendon & Currah 1977), to obstruct stigma penetration by cross-pollen and to negatively affect seed set (Shore & Barrett 1984, Galen *et al*. 1989).

We conclude from our experiments that a post-zygotic mechanism, probably early-acting inbreeding depression, is responsible for the lower seed number matured in self- than in cross-pollinations. While seed number was lower, the number of aborted seeds was higher following self- than following cross-pollinations, indicating post-zygotic processes. Reduced reproductive success upon self-pollination in comparison to cross-pollination was associated with increasing abortion rate in *Kalmia latifolia* (Rathcke & Real 1993), *Vaccinium angustifolium* (Hokanson & Hancock 2000), *V. corymbosum* (Krebs & Hancock 1990, 1991, Hokanson & Hancock 2000), *V. myrtilloides* (Hokanson & Hancock 2000), and *V. myrtillus* and *V. vitis-idaea* (Guillaume & Jacquemart 1999).

Our model on number of aborted *vs*. matured seed in self- and cross-pollinations resulted in a regression coefficient of –0.88 (± SE = 0.137) for bilberry that did not differ significantly from –1 (Fig. 3). When we calculated the same relationship for partially self-incompatible *Campanula rotundifolia* (using data from Nuortila *et al*. 2004), the corresponding regression coefficient was 0.072: self-pollinated flowers in *C. rotundifolia* did not abort more seeds than cross-pollinated flowers. While these results are promising, the method has some obvious limitations. The validity of the model is based on reliable distinction between aborted seeds and unfertilized ovules. First, if ovules are fertilized but aborted

very early without being noticed as aborted, the slope of the regression line would be less negative than –1, the false conclusion could be drawn that abortion was caused by self-incompatibility instead of early-acting inbreeding depression. On the other hand, if unfertilized ovules would enlarge they could be classified as aborted, and the model would indicate inbreeding depression instead of self-incompatibility. Second, the model as such does not distinguish late-acting self-incompatibility from early-acting inbreeding depression. According to Seavey and Bawa (1986), if self-incompatibility was late-acting, the abortion would be active during a certain time/phase of seed development, and therefore aborted seeds would exhibit little variation in size. Since the aborted seeds in bilberry varied in size (data not shown), we have no evidence that late-acting self-incompatibility could confound the present results. Anyhow, due to these limitations, the model should be checked with plant species that are known to be partially self-incompatible and/or species with known inbreeding depression.

The relative survivorship (R) of offspring from self-pollination in relation to offspring from cross-pollination, measured in terms of seed number per fruit, varied between the *V. myrtillus* clones from 0.03 to 0.74. Although this observation is based on one measurement per clone only, it presumably reflects different levels of genetic load. Variation in self-fertility among different individuals has been interpreted as indicating inbreeding depression (Seavey & Carter 1994). A positive relationship between self and outcross-fertility is said to be a further indication of early-acting inbreeding depression, and has been found in two out of three investigated *Vaccinium* species (Krebs & Hancock 1991, Hokanson & Hancock 2000). However, we did not see any significant relationship between the number of mature seeds in self- *vs*. cross-pollinated flowers for our data (Pearson $r = 0.067$, $P > 0.05$, $n = 18$). If inbreeding depression is caused by recessive lethal alleles, then there is not necessarily a relationship between self- and cross-fertility.

Some species show different levels of inbreeding depression at different locations of their distribution. For instance, progenies of Scots pine (*Pinus sylvestris*) from southern populations showed more severe inbreeding depression than did progenies from northern populations, probably as a consequence of more intense purging of deleterious alleles following more intense selfing in the north (Kärkkäinen *et al*. 1996). Since inbreeding depression is typically greater in harsh environments (e.g. Dudash 1990, Frankham *et al*. 2002), inbreeding depression might vary across the geographical range of *Vaccinium myrtillus*. However, our estimation for inbreeding depression ($\delta = 0.8$) for seed number in bilberry in a northern boreal forest is similar to the value that Guillaume and Jacquemart (1999) calculated for *V. myrtillus* ($\delta = 0.84$ at seed maturation) at Upper Ardennes, a central-European population. Although the growth period is shorter in the boreal forest, the bilberry population at Upper Ardennes might grow in an equally harsh environment, with low winter snow cover. Altogether, in those ericaceous species for which there are estimations, the data indicate rather high levels of early-acting inbreeding depression (e.g. $\delta = 0.74$–0.83, Rathcke & Real 1993, Guillaume & Jacquemart 1999, Mahy & Jacquemart 1999). Provided there is some natural self-pollination, why is genetic load so high? In addition to the long lifespan of ericaceous species, at least in *Vaccinium* species recruitment of new individuals into undisturbed vegetation is rather rare (Eriksson & Fröborg 1996, Hautala *et al*. 2001). Hence, there may be low establishment of selfed offspring and subsequently, weak selection against deleterious alleles.

Our estimate of 7.8 lethal equivalents per zygote for *V. myrtillus* is close to the mean value of 9.6 lethal equivalents for tetraploid *V. corymbosum* (Krebs & Hancock 1991). Our estimate lies within the range of values reported for coniferous trees: 10 lethal equivalents for Douglas-fir (*Pseudotsuga menziesii*) (Sorensen 1969), and 6.3 lethal equivalents for Scots pine (Kärkkäinen *et al*. 1996). High estimates have also been reported for other long-lived perennial species (e.g. 11.0 lethal equivalents for *Epilobium obcordatum*, Seavey & Carter 1994). Much lower estimates around one to two lethal equivalents per zygote were calculated for some short-lived herbaceous species (Levin 1984, Lynch & Walsh 1998). Nevertheless, these figures may depend

on the environment in which the study organism was investigated. In some species the estimations for number of lethal equivalents have been lower in captivity or garden experiments than in the wild (Hedrick & Kalinowski 2000).

In summary, the present data support earlier suggestions that inbreeding depression acting at the seed stage may limit seed set in bilberry. Moreover, inbreeding depression in a northern boreal forest was similar to that calculated for a central-European population, and the estimated number of lethal equivalents per zygote was similar to those in other long-lived perennial species. High levels of early-acting inbreeding depression in ericaceous shrubs tend to confirm an association between a plant's growth form (i.e. longevity, clonality) and the magnitude of the genetic load.

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