Investigation of pollen limitation, inbreeding and outbreeding depression and heterosis in *Euphrasia stricta* var. *suecica* on Gotland

Emelie Nilsson

Degree project in biology, Master of science (2 years), 2017
Examensarbete i biologi 30 hp till masterexamen, 2017
Biology Education Centre and Department of Ecology and Genetics/Plant Ecology and Evolution, Uppsala University
Supervisors: Nina Sletvold and Brita Svensson
External opponent: Hampus Petrén
# Index

Abstract ................................................................................................................. 2
Introduction .............................................................................................................. 3
   Aims and predictions .......................................................................................... 5
Materials and methods ........................................................................................... 6
   Species description - E. stricta var. suecica ....................................................... 6
   Field work – pollination treatments ..................................................................... 6
   Laboratory work – weighing and germination .................................................... 7
Statistical tests and analyses .................................................................................. 8
   Effect of bagging and delay ................................................................................ 8
   Autonomous self-pollination, inbreeding and outbreeding depression ............... 8
Results ..................................................................................................................... 8
   Effects of bagging and delayed pollination ........................................................ 9
   Autonomous self-pollination ................................................................................ 11
   Inbreeding and outbreeding depression and heterosis ......................................... 11
   Pollen limitation .................................................................................................. 13
   Number of seeds in relation to flowering date .................................................... 14
Discussion ............................................................................................................... 14
   Autonomous selfing and pollen limitation .......................................................... 15
   Inbreeding and outbreeding depression ................................................................ 15
   Differences between meadows ............................................................................. 16
   Implications for conservation ............................................................................. 17
   Cautions ............................................................................................................... 18
   Conclusions ......................................................................................................... 18
Acknowledgements ................................................................................................. 18
References ............................................................................................................... 19
Appendix 1 .............................................................................................................. 23
Abstract
Habitat fragmentation is one of the main threats to semi-natural grasslands all over Europe, causing reductions in both numbers of populations and numbers of individuals within populations. Small isolated populations are particularly vulnerable to fluctuations that reduces population size and could lead to loss of genetic variation due to inbreeding depression, or fixation of harmful alleles due to genetic drift. The aim of this study was to investigate if the critically endangered eyebright *Euphrasia stricta* var. *suecica* experiences pollen limitation, inbreeding depression and heterosis or outbreeding depression. Low pollen limitation was predicted as well as low inbreeding depression due to high selfing rate. Outbreeding depression or heterosis was expected based on previous studies indicating strong genetic differentiation among populations. This study was conducted in two traditionally managed meadows in the central part of Gotland, Sweden, using supplemental hand-pollinations and controlled crosses. Individuals were collected when the flowering period was over and seeds were counted and weighed. There were no signs of either pollen limitation, inbreeding depression, heterosis or outbreeding depression in any of the two wooded hay meadows. The degree of autonomous selfing was high, indicating that *E. stricta* var. *suecica* is not dependent on pollinators for seed production. A significant difference between the meadows was found in proportion of viable seeds. As there were no signs of local adaptation, individuals could be translocated between the two studied populations or seed sowings could be used to increase population size. However, before such conservation efforts are undertaken, it is important to conduct further studies that investigate several populations including more life-stages.
Introduction

One of the main threats to biodiversity today is habitat fragmentation (Templeton et al. 1990; Young et al. 1996; Jump and Peñuelas 2006; Matthies et al. 2004). A reduction in the number of populations as well as decreasing population sizes are two consequences of habitat fragmentation (Anderson et al. 2011), resulting in more isolated populations that are smaller in size. Environmental, demographic and genetic stochasticity are factors that affect small populations more than larger ones (Matthies et al. 2004). A reduction in population size usually leads to a direct loss of genetic variation, which continues due to increased genetic drift and inbreeding (Matthies et al. 2004). Lower genetic variation could in the short term result in reduced population fitness, and in the long term in reduced evolutionary flexibility, which in turn could lead to difficulties with persisting in a changing environment (Matthies et al. 2004).

The genetic processes associated with fragmentation and small population sizes have been of large interest in plant conservation studies (Ellstrand and Elam 1993). Inbreeding depression is one of these processes, and is the result of a reproduction between two relatives which causes lower fitness in the offspring (Hedrick and Kalinowski 2000; Lundgren et al. 2013; Oakley et al. 2012). Inbreeding depression is due to increased homozygosity (Ellstrand and Elam 1993) which leads to the expression of recessive harmful alleles. Studies have shown that small plant populations have lower inbreeding depression compared to large ones, due to loss of strongly harmful alleles by chance (Oakley et al. 2012). Another process that also reduces genetic variation in small populations is genetic drift that may lead to fixation of mildly deleterious alleles (Oakley et al. 2012).

One way to increase genetic diversity in two or more inbred populations is by crossing individuals between them. Strong genetic differentiation can lead to both heterosis and outbreeding depression when populations are crossed. Heterosis is when crossing between populations result in increased fitness in the offspring compared to crosses within populations, whereas outbreeding depression is the opposite (Oakley et al. 2012). The outcome depends on whether the genetic differentiation is a result of neutral processes like inbreeding or drift, which could lead to fixation of harmful alleles, or of divergent selection, leading to local adaptation. Crosses should lead to heterosis if the population differentiation is a result of inbreeding and drift. The genetic diversity would then be increased because new alleles are added to the population, and it is possible that crosses between populations will perform better than within-population crosses, because of heterosis effects (Hufford and Mazer 2003; Marsden et al. 2013). If the crossed populations are locally adapted, crossings would result in outbreeding depression, where the offspring is not adapted to any of the parental environments and has a lower fitness (Luijten et al. 2002). Neutral genetic differentiation may be correlated with adaptive differentiation, which in the long term could result in outbreeding depression if the genes are adapted to a specific environment (Hufford and Mazer 2003). Adaptive variation at a local scale could reflect habitat differences such as temperature, rainfall, wind exposure, shading, and management regimes. Such differences could therefore to some degree result in locally adapted populations, where crossings between such populations become unsuccessful and could result in outbreeding depression.
Fragmentation of habitats may also have indirect effects on plant populations via interactions with other organisms, especially pollinators (e.g., Dauber et al. 2010). The abundance of pollinators may be lower in fragmented landscapes, due to less resources and longer distances between them. Individuals in small populations should be less attractive to pollinators, leading to the expectation of stronger pollen limitation (PL) in small populations (Becker et al. 2011; Dauber et al. 2010). Several studies have found that small populations are suffering more from pollen limitation compared to large populations (Zhang and Lou 2015; Kolb 2005), due to lower visitation rates. In an experiment investigating if habitat fragmentation could affect the interaction between a plant (Betonica officinalis) and its pollinator it was found that fragmentation caused a reduction in outcrossing rate, leading to lower genetic diversity in the plant population (Rusterholz and Baur 2010). In a review and synthesis by Aguilar et al. (2006) they found a large negative effect of habitat fragmentation on both pollination and sexual reproduction (measured as either seed or fruit production). However, strong and consistent PL can lead to selection for reproductive assurance (RA) and selfing (Kennedy and Elle 2008; Martén-Rodríguez and Fenster 2010; Busch and Delph 2012), and this should be especially strong in annual plants that depend on yearly seed production (Fausto et al. 2001). Most annuals have the ability to self-pollinate compared to perennial plants (Snell and Aarssen 2005).

In cases where local populations have already gone extinct, reintroductions and crosses to attempt to restore genetic variation may be used as conservation tools (Daws and Koch 2015). Genetic restoration could be one solution to avoid extinction by translocating individuals between related populations, to increase genetic diversity in both populations (Hedrick and Kalinowski 2000). However, if populations express strong local adaptation, the success of reintroductions and genetic rescue programs may be low. Introduced plants may be poorly adapted to the new local conditions, and novel genes might disrupt existing local adaptation, leading to reductions in population fitness (i.e., outbreeding depression). To design optimal restoration strategies, it is therefore crucial to quantify the extent and spatial scale of local adaptation (cf. Vergeer et al. 2004). In a meta-analysis by Leimu and Fischer (2008) they concluded that local adaptation is expected to be restricted in small populations because selection should be less effective because of less genetic variation for selection to work on. They also concluded that small populations therefore face a harder challenge to cope with changes in the environment (Leimu and Fischer 2008).

Semi-natural grasslands is a habitat type that is currently experiencing extensive fragmentation, and are currently threatened across the whole of Europe, due to changes in land use (Sala et al. 2000). For example, Jantunen (2003) investigated habitat fragmentation in southern Finland and found that both the number and area of semi-natural grasslands has decreased enormously due to intensification of agriculture or abandonment. Semi-natural grasslands are typically species-rich habitats dominated by plants with low competitive ability and high tolerance to disturbances (Öster 2006). Many of them are short-lived perennials or annuals. Such disturbance-dependent species are often outcompeted when traditional land use is abandoned, and some kind of management, for example mowing, is usually introduced to increase plant population viability.

In Sweden, the island Gotland in the Baltic Sea represents a highly fragmented landscape of semi-natural grasslands. On Gotland, wooded hay meadows are very important areas containing a high diversity of plants, including many annuals. Eyebrights (the genus
Euphrasia are annual herbs that are strongly connected to managed meadows (Svensson and Carlsson 2013). For their persistence they are in need of repeated management with raking, mowing and grazing every year (Svensson and Carlsson 2013). During the 1900’s many of the wooded hay meadows lost their importance of producing winter forage to the animals (Martinsson 2012) due to increased fertilizing of fields, but also the increasing use of other management tools such as agricultural machines. The number and size of semi-natural grasslands has been strongly reduced, due to changes in land use by transforming them into cropland (Jantunen 2003).

Euphrasia stricta is the most common species in this genus in Sweden, and it is divided into five different varieties (Kolseth and Lönn 2005). Euphrasia stricta var. suecica is one of them. It is red listed as endangered, and the variety grows only on Gotland (Johansson 2007). It is an early-flowering variety that is believed to have evolved on Gotland as a response to mowing in the middle of the summer, due to selection for rapid completion of the life cycle (Karlsson 1984). This makes it unique and is one reason why it should be preserved. E. stricta var. suecica was previously a common species in mown meadows but has declined rapidly in response to change in land-use (Ohlsson 2006). Today, only seven localities remain on Gotland (Artportalen 2017) with populations ranging from only two individuals in one place up to 9000 individuals in another wooded hay meadow (Figure 1). Several species in the genus are known to be highly selfing (Gomez 2002; Kolseth and Lönn 2005), and a low genetic diversity in populations of E. stricta var. suecica also suggests a high selfing rate (Kolseth and Lönn 2005). There is strong genetic differentiation among populations in neutral markers (mean $F_{ST} = 0.60$), indicating weak gene flow and strong drift in all varieties of E. stricta. Populations of E. stricta var. suecica growing close to each other tend to differ more than if they are growing a longer distance apart (Kolseth and Lönn 2005). This is opposite to the isolation by distance (IBD) model (Epperson 2007), and suggests a history of `random´ gene flow, where gene flow is no more likely among close populations than among distant ones. This could reflect the historical way of taking care of the hay. Perhaps hay containing E. stricta var. suecica seeds was transported with people or agricultural equipment from one meadow to another but that the meadows were not necessarily close to each other.

Like other eyebrights, E. stricta var. suecica lacks a seedbank (Johansson 2007), and population dynamics should thus be highly dependent on annual seed production. However, it is not known to what degree seed production is pollen limited, or whether self-pollination or outcross pollination are dominating.

**Aims and predictions**

The aims of this project were to establish whether Gotland populations of E. stricta var. suecica are pollen limited, and whether there is any evidence of inbreeding depression and/or outbreeding depression or heterosis. Due to the variety’s status as critically endangered, I chose to work in the two largest known populations, Anga prästänge (15 000 individuals) and Bendes strandäng (32 000 individuals; Johansson 2007), separated by 5 km.

Supplemental hand-pollination at the whole-plant level was used to quantify pollen limitation (cf. Sletvold et al. 2010) and controlled crossings within and between the populations were done to quantify inbreeding and outbreeding depression and heterosis (cf. Sletvold et al. 2012).
I expected weak pollen limitation and a high degree of self-fertilization in the populations, which should result in weak inbreeding depression. I also expected that the strong genetic differentiation should result in heterosis (if caused by drift and random fixation of harmful alleles) or outbreeding depression (if reflecting local adaptation).

**Materials and methods**

The study was conducted in two different wooded hay meadows in the midmost part of Gotland (Fig. 1), Anga prästänge and Bendes strandänge. Both meadows are known as areas containing the largest populations of *E. stricta var. suecica*. Anga prästänge is a small wooded hay meadow located close to a large road. It is a Natura 2000 area in EU’s network (Petersson and Ingmansson 2007) and is owned by the church (Ohlsson 2006). Bendes strandänge is an open wooded hay meadow with scattered trees close to the shoreline. Such wooded meadows are not common anymore (Ohlsson 2006). Both of the meadows are mown after the 15th of July every year, and grazed afterwards (Martinsson 2012).

*Species description - E. stricta var. suecica*

Eyebrights are semi-parasites, also called hemi-parasites, meaning that they have their own photosynthesis but they also obtain nutrients from their host. One of the hosts for *E. stricta var. suecica* is red clover (*Trifolium pratense*), but also meadow grass (*Poa pratensis*) and red fescue (*Festuca rubra*) are known hosts (Svensson and Carlsson 2004). Seeds germinate in spring and the plants flower in mid-June. Flowers are self-compatible, and are visited by bumblebees and small flies (French et al. 2005). The relative importance of selfing versus outcrossing for seed production is unknown. Each individual of the eyebrights produces maximum 16 seeds in each capsule (Svensson and Carlsson 2013). The seeds mature in July and are spread at short distances by wind. Seeds only survive for up to two years in the soil, and the very short-lived seed bank makes it important to produce many seeds every year (Almqvist 2016). *E. stricta var. suecica* is sensitive to shading, and dependent on mowing and removal of litter each season (Ekstam and Forshed 1992; Karlsson 1982).

*Field work – pollination treatments*

In both meadows, 175 individuals were individually marked with a numbered flag at the start of the flowering period. The individuals were randomly divided into five treatment...
groups of equal size: 35 with no pollination (pollinators excluded by enclosing individuals in a bag made of mosquito net = Bag), 35 open-pollinated (natural pollinator visitation = Open), 35 self-pollinated by hand (Self), 35 outcross-pollinated by hand with pollen from another individual in the same population (located at least 1 meter away; Outcross-within), and 35 outcross-pollinated by hand with pollen from the other population (Outcross-between).

In Anga prästänge, 10 additional individuals were both bagged and self-pollinated by hand (Self+Bag) to investigate whether the bagging treatment per se had any effect. Finally, to investigate potential negative effects of the pollination-delay that occurred when pollen was transported from one meadow to the other in the between-population outcrossing-treatment, 15 additional individuals in Anga were outcross-pollinated with a delay of 30-60 minutes (the time it took to transport pollen between meadows) with pollen from the same population (Delay).

Treatments were conducted at the whole-plant level, meaning that all flowers on an individual were pollinated. Pollination was done by using a small brush. Whole anthers were moved between individuals by using the brush. Anthers were first put on the pistil and pollen was mixed and left in the flower. Pollinations were conducted from 13th of June until all flowers had been pollinated, 8th of July. Pollination was repeated several times on the same individual to ensure that all flowers had been pollinated at least once. For each individual, independent of treatment, the number of open flowers was counted every time the plant was visited. The date for the first flower in bloom was also noted. During the field study, few potential pollinators visiting *E. stricta var. suecica* flowers were observed. Seed predators of unknown *Diptera* species were found in some of the capsules (determination made by Hans Mejlon), but were too undeveloped to allow further determination. On some of the flowers larvae from the orders *Coleoptera* and *Lepidoptera* were found, which might be some of the pollinators.

The time needed for setting seeds was approximately one month. Before the mowing took place in the middle of July all marked individuals (375 individuals in total) were collected and stored in paper bags closed with staples. Individuals were collected when the first capsule had opened and mature seeds were visible. This was done during the whole field period. All bags were then dried in room temperature for one month. Afterwards, bags were placed in a paper box and stored for two months until the counting of seeds started.

*Laboratory work – weighing and germination*

Dry plants were taken into the lab to determine estimates of female fitness. The number of fruits was counted and the total number of seeds per individual was noted. All seeds per individual were pooled and weighed. The total seed weight was divided by the total number of seeds to get a mean individual seed weight. Seeds were put in a freezer from 1/12 2016 until 12/1 2017 (42 days) for stratification.

A germination experiment was conducted to investigate the effect of crossing treatment on seed viability. The experiment started after seed dormancy had been broken by the stratification. Twenty seeds from 30 individuals from four of the treatments (Bag, Open, Outcross-within and Outcross-between) were used. To increase germination success, only seeds that were expected to germinate were selected from each individual, these were seeds that were swollen, with a brown/beige color and white stripes. Wrinkly seeds that looked empty inside and with a yellow to white color were classified as undeveloped/aborted. The
proportion of potentially viable seeds (number of viable seeds divided by total number of seeds) was calculated for all individuals.

Seeds were sown in January 2017 (20/1-22/1) on filter papers in petri dishes, and 1.5 ml tap water was added. All petri dishes were placed randomly in a growth chamber with a light intensity of 152-162 µE, light for 18 hours (between 6.00 and 24.00), and temperature ranging between 4 and 12°C during night, and between 8 and 20°C during day. Seeds were watered when necessary until the experiment was ended after 4 weeks in the growth chamber.

Temperature conditions were chosen based on a similar experiment with two other species in the *Euphrasia* genus (Liebst and Schneller 2008).

Only 2 seeds germinated during the 4 weeks of the experiment (both from the Outcross-between treatment) and therefore no statistical tests were done to investigate the effect of crossing treatment on seed viability. After all measurements and tests were finished in the lab, the seeds were returned to the paper bags and were sown (all seeds except those from the between-population outcrossing) into the meadows they came from in April 2017, not to affect the already sensitive populations. Seeds from the Outcross-between treatment will not be sown due to an agreement with the County administration board at Gotland. If local adaptations exists in the two different wooded hay meadows, seeds should not be sown to prevent dispersal of foreign genes in each area.

**Statistical tests and analyses**

*Effect of bagging and delay*

To test if bagging per se had any effect on seed production and/or seed weight, the self-pollination treatments with and without bagging (Self vs. Self+bag) were compared in a t-test. To test for an effect of delayed pollination, the within-population outcrossing treatments (Delayed vs. Outcross-within) were tested in a t-test with the same response variables as above (number of seeds and seed weight).

*Autonomous self-pollination, inbreeding and outbreeding depression*

To test for effects of pollination treatment and meadow on number of fruits with seeds, number of seeds and seed weight, I used a two-way ANOVA including the fixed factors treatment (five levels) and meadow (two levels). From pairwise treatment comparisons the degree of self-pollination (Bag vs. Open), inbreeding depression (Self vs. Outcross-within), outbreeding depression or heterosis (Outcross-within vs. Outcross-between), and pollen limitation (Open vs. Outcross-within) was determined.

The proportion of viable seeds were tested in a Generalized Linear Mixed Model (GLMM), including the fixed factors meadow and treatment. Plant identity was included as a random effect. The model used a binomial error distribution.

To test whether earlier flowering was associated with higher seed production, I used a two-way ANOVA including the factors flowering date (four levels) and meadow, and the interaction between them.

All analyses were performed using the statistical program R (R Core Team 2016).

**Results**
**Effects of bagging and delayed pollination**

Plants that were self-pollinated and bagged had on average 7% more seeds that weighed 18% less than self-pollinated plants that were left in the open, but there was no statistically significant effect of treatment on either the number of seeds ($t = -0.19$ df = 10.46; $p = 0.86$; N = 44; Fig. 2A) or seed mass ($t = 1.99$; df = 20.46; $p = 0.06$; N = 44; Fig. 2B).

**Figure 2.** The effect of bagging on (A) number of seeds (Mean±SD, Self+Bag = 45.40 ± 46.19 Self = 42.59 ± 23.94) and (B) seed weight (Self = 0.00021 ± 0.000068, Self+Bag = 0.00017 ± 0.000048) in Anga prästänge.

Likewise, the delayed pollination did not significantly affect the number of seeds ($t = 0.51$; df = 21.84; $p = 0.62$; N = 48; Fig. 3A) or the seed weight ($t = 1.5$; df = 26.04; $p = 0.15$; N = 48;
Fig. 3B). On average, the individuals with delayed outcrossing had 9% fewer seeds that weighed 13% less than the ones with ordinary outcrossing.

Figure 3. The effect of delayed pollination on (A) number of seeds (Mean ± SD), (Outcross-within = 53.73 ± 28.29, Delayed = 48.27 ± 36.80) and (B) seed weight (Outcross within = 0.00019 ± 0.000048, Delayed = 0.00016 ± 0.000051) in Anga prästänge.
Since none of the control-treatments (Self+Bag or Delayed) had any significant effect on the number of seeds or the seed weight, they were excluded from the following analyses.

**Autonomous self-pollination**
Individuals in both populations produced as many fruits and seeds when pollinators were excluded as when they were open-pollinated (Bag vs. Open treatment; Table 1, Fig. 4 and 5). Neither were there any effect of pollinator exclusion on seed weight (Fig. 5) or the proportion of viable seeds produced (Fig. 7).

**Inbreeding and outbreeding depression and heterosis**
There were no significant inbreeding depression (Self vs. Outcross-within treatment) or outbreeding depression or heterosis (Outcross-within vs. Outcross-between treatment) in the two wooded hay meadows (Table 1). Number of fruits with seeds (Fig. 4), number of seeds (Fig. 5), seed weight (Fig. 6), and proportion of viable seeds (Fig. 7) did not significantly differ among the crossing treatments (Table 1 and 5). The proportion of viable seeds was significantly lower in the Bendes meadow compared to the Anga meadow (Table 5, Fig. 7).

Table 1. ANOVA table. The effects of meadow (Anga and Bendes), crossing treatment (Bag, Open, Self, Outcross-within, Outcross-between), and their interaction on number of fruits with seeds, number of seeds and seed weight in *Euphrasia stricta var. suecica* analyzed with two-way ANOVA.

<table>
<thead>
<tr>
<th></th>
<th>Sum sq</th>
<th>Df</th>
<th>F value</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of fruits with seeds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meadow</td>
<td>35.7</td>
<td>1</td>
<td>2.43</td>
<td>0.12</td>
</tr>
<tr>
<td>Treatment</td>
<td>62.3</td>
<td>4</td>
<td>1.06</td>
<td>0.38</td>
</tr>
<tr>
<td>Interaction</td>
<td>15.7</td>
<td>4</td>
<td>0.27</td>
<td>0.90</td>
</tr>
<tr>
<td>Residuals</td>
<td>5006.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Number of seeds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meadow</td>
<td>2085</td>
<td>1</td>
<td>1.33</td>
<td>0.25</td>
</tr>
<tr>
<td>Treatment</td>
<td>5873</td>
<td>4</td>
<td>0.93</td>
<td>0.44</td>
</tr>
<tr>
<td>Interaction</td>
<td>2452</td>
<td>4</td>
<td>0.39</td>
<td>0.82</td>
</tr>
<tr>
<td>Residuals</td>
<td>534570</td>
<td>340</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean weight seeds (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meadow</td>
<td>8.010e-09</td>
<td>1</td>
<td>1.41</td>
<td>0.24</td>
</tr>
<tr>
<td>Treatment</td>
<td>2.44e-08</td>
<td>4</td>
<td>1.07</td>
<td>0.37</td>
</tr>
<tr>
<td>Interaction</td>
<td>1.35e-08</td>
<td>4</td>
<td>0.60</td>
<td>0.67</td>
</tr>
<tr>
<td>Residuals</td>
<td>1.93e-06</td>
<td>340</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4. Number of fruits with seeds (mean ± SD) for each treatment in both meadows, Anga and Bendes.

Individuals in Bendes meadow had on average 4% more fruits with seeds across treatments (Table A2, Appendix 1) but the difference was not significant (Table 1).

Figure 5. Number of seeds (mean ± SD) for each treatment in both meadows, Anga and Bendes.

Individuals in Anga had on average 30% more seeds if treatments were pooled (Table A3 Appendix 1, Fig. 5). The difference was however not significant (Table 1).

Figure 6. Seed weight (mean ± SD) for each treatment in both meadows, Anga and Bendes.
Mean difference in seed weight between meadows was 12% if all treatments were pooled. Anga was the meadow that tended to have heavier seeds (Fig. 6, Table A4 Appendix 1).

![Proportion of viable seeds](image)

Figure 7. Proportion of good seeds for each treatment (mean ± SD).

Table 5. Summary of GLMM (Generalized Linear Mixed Model), Binomial distribution of data. Effects of meadow (two levels) and treatment (four levels) on proportion of good seeds in *Euphrasia stricta var. suecica*. N (Anga, Bendes) = Bag: 34, 34; Open: 32, 34; Outcross between: 33, 35; N = Outcross within: 30, 35.

| Proportion of viable seeds | Estimate | Std. Error | z value | Pr(>|z|) |
|---------------------------|----------|------------|---------|---------|
| Meadow B                  | -1.61    | 0.53       | -3.05   | 0.0020  |
| Treatment Open            | -0.10    | 0.54       | -0.19   | 0.85    |
| Treatment Outcross between| -0.38    | 0.54       | -0.72   | 0.47    |
| Treatment Outcross within | -0.52    | 0.55       | -0.94   | 0.35    |
| Interaction: Meadow B -Treatment Open | -0.19 | 0.75     | -0.25   | 0.80    |
| Interaction: Meadow B -Treatment Outcross between | -0.09 | 0.74 | -0.12 | 0.90 |
| Interaction: Meadow B - Treatment Outcross within | -0.13 | 0.74 | -0.18 | 0.86 |

**Pollen limitation**

The populations in Anga and Bendes were not pollen limited (Table 1). No significant difference was found between the open-pollinated and supplementally hand-pollinated treatments (Open vs. Outcross-within) or between the meadows in any response variable. No significant interaction was found between meadow and treatment.
**Number of seeds in relation to flowering date**

Individuals in Anga started to flower significantly earlier than those in Bendes, but there was no effect of flowering date on number of seeds produced (Table 6).

![Flowering date vs. number of seeds](image)

**Figure 8.** The effect of flowering date on the number of seeds (mean±SD).

**Table 6.** ANOVA table. Effects of meadow (Bendes and Anga) and flowering date (4 levels) on number of seeds produced analysed with two-way ANOVA.

<table>
<thead>
<tr>
<th>Number of seeds</th>
<th>Df</th>
<th>Sum Sq</th>
<th>Mean Sq</th>
<th>F value</th>
<th>Pr (&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meadow</td>
<td>1</td>
<td>12647</td>
<td>12646.7</td>
<td>8.26</td>
<td>0.004</td>
</tr>
<tr>
<td>Date</td>
<td>3</td>
<td>9873</td>
<td>3290.9</td>
<td>2.15</td>
<td>0.094</td>
</tr>
<tr>
<td>Interaction: Meadow-Date</td>
<td>2</td>
<td>158</td>
<td>79.2</td>
<td>0.05</td>
<td>0.95</td>
</tr>
<tr>
<td>Residuals</td>
<td>329</td>
<td>503810</td>
<td>1531.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Discussion**

There was no evidence of pollen limitation, inbreeding depression or outbreeding depression or heterosis in any of the two studied meadows of the endangered *E. stricta var. suecica* (Table 1). My results suggest that the species is highly selfing and not dependent on pollinators for maximizing seed production. The results also suggest that 1) low population viability is not due to lack of sufficient pollination, 2) there is a history of high selfing rates and 3) the specific seed source may be of minor importance for reinforcements and reintroductions. Altogether, conservation should focus on using the optimal management actions (raking, mowing and aftermath grazing), maybe combined with reinforcements.
Autonomous selfing and pollen limitation

My results indicate that autonomous self-pollination is the most important way of reproduction in *E. stricta* var. *suecica*. When individuals with pollinators excluded were compared with open-pollinated individuals, there was no difference in either the number of fruits, the number of seeds or the seed weight. Neither was there any effect of supplemental hand-pollination. This suggests that both studied populations are highly selfing, and do not depend on pollinators for maximizing seed production. This is consistent with predictions based on previous population genetic analyses that documented very low genetic variation within populations of *E. stricta* var. *suecica* (Kolseth and Lönn 2005). Comparable results have been found for another species in this genus, *Euphrasia willkommii* (Gómez 2002), where seed production was similar if pollinators were excluded as if they were not. Kennedy and Elle (2008) also found almost identical results when investigating the importance of reproductive assurance (RA) in the annual plant *Collinsia parviflora*. RA is expected to be selected for if populations become small and there are few individuals to mate with, or if pollinators become rare or habitats too fragmented (Kennedy and Elle 2008). This should be especially important in annual species that are highly dependent on seed production in a single year. In an experimental study, Bodbyl Roels and Kelly (2011) compared populations of the annual *Mimulus guttatus* with and without pollinators and found that populations where insect pollination was excluded at first suffered in fitness, but after a few generations they rebounded as they evolved a high degree of self-fertilization. The annual *E. stricta* var. *suecica* might also have evolved rapidly to become self-fertilizing, as a result of pollen limitation.

Alternatively, the documented ability of selfing and lack of pollen limitation in both *E. stricta* var. *suecica* and other species in the genus may reflect that in the history of *Euphrasia*, pollinators have been a limiting factor at an earlier time, resulting in the evolution of a mating system with a high degree of selfing that was present before *E. stricta* var. *suecica* evolved. Low pollinator visitation seems to be true today as well, because very few pollinators were observed during the flowering period in the two populations in this study. This suggests that pollinators are not attracted to the study populations, perhaps because they are too small, or because other co-flowering species are more attractive.

Inbreeding and outbreeding depression

In both wooded hay meadows, individuals that were self-pollinated had as high fitness as those outcrossed with pollen from the same population. This shows that none of the studied populations of *E. stricta* var. *suecica* suffer from inbreeding depression. At least, inbreeding does not affect fitness components at early life stages, such as number of fruits, number of seeds or seed weight. This is in line with what was found in the forest herb *Geum urbanum* (Vandepitte et al. 2010). Since seeds did not germinate I could not examine whether inbreeding depression occurs at later life stages.

The lack of inbreeding depression may reflect a long history of selfing in *E. stricta* var. *suecica* which may have purged the genetic load and reduced inbreeding depression (Husband and Schemske 1996). However, this process is not expected to be effective in small populations (Byers and Waller 1999). It is perhaps more likely that genetic drift and inbreeding has led to high homozygosity and fixation of mildly deleterious alleles in the populations (Whitlock et al. 2000). Small populations should lose heterozygosity faster than larger populations, and as the inbreeding in the two studied populations is likely to be
continuous, the frequency of heterozygosity should end up close to zero (Ellstrand and Elam 1993). In such highly inbred and homozygous populations, the outcrossing will be nearly the same as selfing, and the lack of inbreeding depression is not surprising.

Before this study started it was known that populations of *E. stricta* var. *suecica* are strongly differentiated at neutral loci (Kolseth et al. 2005). This is likely to reflect strong inbreeding and genetic drift, with very little gene exchange among populations. If different mildly deleterious alleles have become fixed in the populations (Whitlock et al. 2000), crossings between populations should lead to heterosis (Oakley et al. 2012). In another annual plant (*Leavenworthia alabamica*) a small amount of heterosis was found (Busch 2006) and Hauser and Loeschcke (1994) found heterosis in offspring in the perennial plant, *Lychnis flos-cuculi*. However, no heterosis was found in any of the two studied *E. stricta* var. *suecica* populations, which suggests that maybe it is the same harmful alleles that are fixed in both of the populations.

Kolseth et al. (2005) found that the genetic difference in *E. stricta* var. *suecica* was higher between populations that are close to each other than populations that are further apart. This is opposite to the pattern predicted by isolation by distance (Epperson 2007), and could reflect local adaptation to local habitat differences. Therefore, outbreeding depression could be expected if the strong population differentiation at neutral markers is correlated with adaptive differentiation (Kolseth and Lönn 2005). However, the lack of outbreeding depression suggests no local adaptation in any of the populations at least when concerning seed set. Previous studies have documented outbreeding depression at different spatial scales from among sub-populations (Sletvold et al. 2012) to populations across countries (Matter et al. 2014).

**Differences between meadows**

The proportion of viable seeds significantly differed between the two studied wooded hay meadows (Fig. 7, Table 5). Bendes strandängÄ¥ had a lower proportion of good seeds compared to Anga prästänge (Table 6). Flowers in Bendes started to flower two days later than those in Anga which in turn resulted in less time to mature seeds since all individuals were collected at the same time in both meadows. The location of Bendes strandängÄ¥ close to the sea could delay flowering and seed maturation due to a colder and windier climate close to the Baltic Sea. In further studies it could be of interest to measure ground temperature to see if there are any differences between the wooded hay meadows.

The number of seeds produced tended to be higher for both meadows at the first date of flowering. *E. stricta* var. *suecica* is already flowering earlier than other *Euphrasia* species except for *Euphrasia stricta* var. *tenuis* which is also an early-flowering variety (Kolseth et al. 2005), but an even earlier flowering does not seem to be selected for, maybe because of low temperatures earlier in the summer.

Germination of seeds did not work that successfully, which could indicate that it is hard to break seed dormancy of *E. stricta* var. *suecica* seeds. This is in line with earlier seed sowing experiments in field populations (Johansson 2007). As mentioned in Liebst and Schneller (2008) germination experiments with *Euphrasia* species depend strongly on the experimental design. Germination of *E. stricta* var. *suecica* seeds in the greenhouse seems to be challenging, which is why conservation should be performed in its native environment where the conditions for germination is right. This should be done by moving the hay within
meadows, to increase the dispersal of seeds. Previous studies of *Euphrasia* species suggest that the traditional management should continue to reduce the amount of litter and by this increase the germination success (Karlsson 1982).

**Implications for conservation**

*E. stricta* var. *suecica* is a rare plant with decreasing numbers of populations and individuals (Johansson 2007), and it is important to find the best way to conserve the species. In addition, the variety is believed to have evolved on Gotland due to mowing in the middle of the summer (Karlsson 1984), why it is unique and should be preserved. Moreover, by conserving eyebrights you also conserve its habitat and the environment for many other species at the same time.伍ed hay meadows are known as areas containing high biodiversity, and Svensson and Carlsson (2013) found that a higher number of *E. stricta* var. *suecica* individuals was associated with a higher number of other species. If at least one species in such a habitat is worth conserving a whole habitat type is conserved and taken care of at the same time.

As predicted, *E. stricta* var. *suecica* is highly selfing and it seems that supplemental hand-pollination does not increase seed production or seed size, suggesting that a lack of pollinators is not a problem. This suggests that attempts to increase seed production by hand-pollination cannot be used as a conservation tool. Lundgren et al. (2013) concluded that some plant species may not suffer as much as predicted from a pollinator decline if they are not as dependent on pollinators. Species that are not limited by low pollinator number could stand even lower amounts of pollinators in the future, because they are highly self-compatible. This is an important point for conservation and management of *Euphrasia stricta* var. *suecica*.

The fact that no effects (in either direction) of crossings between populations were found, might be good in terms of reinforcements and introductions, because it suggests that there is no need to consider where seeds come from. This could also be problematic when thinking about genetic rescue if you cannot increase population fitness by introducing new alleles. It is known that yearly population sizes of *E. stricta* var. *suecica* can vary dramatically (Martinsson 2012) and this has probably to do with weather conditions in the present year as well as seed production the previous year (Svensson, pers. comm.). Because of the loss of genetic variation in inbred populations such populations might struggle when facing both biotic and abiotic environmental changes (Ellstrand and Elam 1993). It is therefore of great importance to manage the wooded hay meadows in the same way as it has been done back in history to avoid negative effects of changes in management and climate.

What is important for *E. stricta* var. *suecica* is that seed production is high every year (Karlsson 1982). Seeds need to mature before mowing, and aftermath grazing or a second mowing is important for germination (Svensson and Carlsson 2004). As an alternative to aftermath grazing Svensson and Carlsson (2013) showed that a second mowing also increases the number of individuals next year. In situ conservation should be used for this species (Ellstrand and Elam 1993). As for many other plant species it is more favorable to conserve this species in its natural habitat. It is a sensitive species and sowing experiments did not work successfully (Johansson 2007) which strengthens that in situ conservation should be done. Management also opens patches with bare soil which stimulates seed germination (Ekstam and Forsshed 1992).
Kolseth and Lönn (2005) concluded that each individual population needs to be conserved and treated as evolutionary units because of the strong population differentiation. However, the results from this study do not indicate any local adaptation in *E. stricta var. suecica*, at least not for the two studied populations, and suggest that it could might be easier to conserve these two populations by exchanging seeds or seedlings between them. If one of the meadows are low in number of individuals one year, one could move seedlings from the other meadow to increase the number of individuals the coming year, which could be comparable with the source and sink-function in meta-populations. However, several sowing experiments have been done without any great success (Johansson 2007), therefore it is perhaps better to leave the plants where they are and give them the best possible conditions to reproduce in their local population.

**Cautions**
The flower morphology of the genus *Euphrasia* is clearly adapted to insect pollination (Karlsson 1971), but during the field work in this study very few pollinators were observed, and open-pollination or hand-pollination did not increase seed production relative to pollinator exclusion. Since none of the pollination-treatments showed any significant differences, one possibility is that hand-pollinations did not work properly. Was the timing wrong for the pollination? In the beginning of the flowering period individuals are only available for cross-pollination because the anthers are not developed yet (Karlsson 1971). Crossings might have worked better if the same flowers had been pollinated a few days in a row to let the different flower parts completely develop. Alternatively, the timing of the pollination was right, but the quality of the pollen was low. Since it is known that eyebrights open their anthers later than the pistil (Karlsson 1971), it might be that the anthers used were not fully mature.

My results could also be specific to these two populations. It is possible that other populations of *E. stricta var. suecica* is suffering from all of these mentioned factors (pollen limitation, inbreeding and/or outbreeding depression), or that effects may be expressed at later life stages.

**Conclusions**
The two studied populations were not pollen limited, and did not express any inbreeding or outbreeding depression or heterosis, quantified at the seed stage. This suggests that efforts to increase pollination success are unlikely to be important. My results indicate that it should be fine to take seeds from a large population to reinforce small populations. However, studies including all existing populations should be conducted to test the generality of the findings. It is also important to be able to get the seeds to germinate, to be able to look at effects across the whole life cycle.

**Acknowledgements**

Nina Sletvold for good supervising, Brita Svensson for suggestions about germination and for information about the species, Linus Söderquist for R and ANOVA help, Mattias Vass for help in the lab with the growth chamber and for good suggestions, Kerstin Jeppsson for introduction to the lab and materials, Giulia Zacchello for good suggestions about seed germination, Matthew Tye for help in R.
References


Lantmäteriet. 2014. Översiktskartan. Skala 1: 100 000. [https://www.lantmateriet.se/sv/Kartor-och-geografisk-information/Kartor/oversiktskartan1/]. Date visited 13 June 2017.


Appendix 1

Table A2. Mean values for number of fruits with seeds. Both meadows together.

<table>
<thead>
<tr>
<th>Meadow</th>
<th>Treatment</th>
<th>Mean number of fruits with seeds</th>
<th>Standard deviation (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anga</td>
<td>Bag</td>
<td>5.24</td>
<td>2.86</td>
</tr>
<tr>
<td>Benes</td>
<td>Bag</td>
<td>6.88</td>
<td>4.88</td>
</tr>
<tr>
<td>Anga</td>
<td>Open</td>
<td>5.28</td>
<td>2.65</td>
</tr>
<tr>
<td>Benes</td>
<td>Open</td>
<td>6.24</td>
<td>4.70</td>
</tr>
<tr>
<td>Anga</td>
<td>Self</td>
<td>4.03</td>
<td>1.95</td>
</tr>
<tr>
<td>Benes</td>
<td>Self</td>
<td>5.59</td>
<td>3.59</td>
</tr>
<tr>
<td>Anga</td>
<td>Outcross within</td>
<td>5.30</td>
<td>2.38</td>
</tr>
<tr>
<td>Benes</td>
<td>Outcross within</td>
<td>6.43</td>
<td>4.37</td>
</tr>
<tr>
<td>Anga</td>
<td>Outcross between</td>
<td>5.94</td>
<td>3.71</td>
</tr>
<tr>
<td>Benes</td>
<td>Outcross between</td>
<td>6.20</td>
<td>4.66</td>
</tr>
</tbody>
</table>

Table A3. Mean values for number of seeds. Both meadows together.

<table>
<thead>
<tr>
<th>Meadow</th>
<th>Treatment</th>
<th>Mean No. Seeds</th>
<th>Standard deviation (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anga</td>
<td>Bag</td>
<td>52.24</td>
<td>37.09</td>
</tr>
<tr>
<td>Benes</td>
<td>Bag</td>
<td>49.71</td>
<td>44.73</td>
</tr>
<tr>
<td>Anga</td>
<td>Open</td>
<td>56.62</td>
<td>36.63</td>
</tr>
<tr>
<td>Benes</td>
<td>Open</td>
<td>39.12</td>
<td>37.92</td>
</tr>
<tr>
<td>Anga</td>
<td>Self</td>
<td>42.59</td>
<td>23.94</td>
</tr>
<tr>
<td>Benes</td>
<td>Self</td>
<td>35.24</td>
<td>29.51</td>
</tr>
<tr>
<td>Anga</td>
<td>Outcross within</td>
<td>53.73</td>
<td>28.29</td>
</tr>
<tr>
<td>Benes</td>
<td>Outcross within</td>
<td>39.74</td>
<td>38.51</td>
</tr>
<tr>
<td>Anga</td>
<td>Outcross between</td>
<td>61.38</td>
<td>58.32</td>
</tr>
<tr>
<td>Benes</td>
<td>Outcross between</td>
<td>41.83</td>
<td>45.62</td>
</tr>
</tbody>
</table>

Table A4. Mean values for seed weight. Both meadows together.

<table>
<thead>
<tr>
<th>Meadow</th>
<th>Treatment</th>
<th>Mean weight (g)</th>
<th>Standard deviation (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anga</td>
<td>Bag</td>
<td>0.00020</td>
<td>6.67E-05</td>
</tr>
<tr>
<td>Benes</td>
<td>Bag</td>
<td>0.00015</td>
<td>7.63E-05</td>
</tr>
<tr>
<td>Anga</td>
<td>Open</td>
<td>0.00020</td>
<td>4.29E-05</td>
</tr>
<tr>
<td>Benes</td>
<td>Open</td>
<td>0.00016</td>
<td>8.16E-05</td>
</tr>
<tr>
<td>Anga</td>
<td>Self</td>
<td>0.00021</td>
<td>6.77E-05</td>
</tr>
<tr>
<td>Benes</td>
<td>Self</td>
<td>0.00015</td>
<td>6.89E-05</td>
</tr>
<tr>
<td>Anga</td>
<td>Outcross within</td>
<td>0.00019</td>
<td>4.84E-05</td>
</tr>
<tr>
<td>Benes</td>
<td>Outcross within</td>
<td>0.00016</td>
<td>8.53E-05</td>
</tr>
<tr>
<td>Anga</td>
<td>Outcross between</td>
<td>0.00021</td>
<td>4.87E-05</td>
</tr>
<tr>
<td>Benes</td>
<td>Outcross between</td>
<td>0.00016</td>
<td>7.78E-05</td>
</tr>
</tbody>
</table>