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Abstract

Changing climate affects the dispersal and phenology of plants and pollinators. Particularly in the spring, climate warming accelerates the timing of flowering and insect emergence. Temporal mismatch is a fairly well studied biological phenomenon. Nevertheless the phenomenon has been studied only for a small number of systems and organisms, and more empirical evidence to show that mismatch occurs and how it is affected by changes in environment. The lack of studies that directly address the effects of this type of phenological mismatches on plant seed set makes it difficult to predict how plant reproductive output will be affected. Although it is reasonable to assume that the reduced seed set is a general response. This response varies greatly among species, which highlights the need for better empirical data. In current the study, the aim is to investigate how the microclimate and flowering time can have an impact on the seed set. The effect of the pollinator on the seed production in a wild flower (*Anemone nemorosa)* was measured at two different flowering times. Three treatments (a) self-pollination (b) hand-pollination and (c) wild-pollination, were applied during both flowering times: one in mid of April (early spring) and the other in early May (late spring). Pollinator abundance was measured at all the experimental sites and the seed sets were counted in collected flowers. The results show that the flowering time has significantly affected the fertile seed production. However microclimate did not had significant effect fertile seed production. Moreover, during the both flowering times, the variation in the abundance and diversity of the pollinator were not detected, thus the microclimate has an effect on the abundance and diversity of pollinator. There was no significant relationship between the pollinator diversity, abundance and seed set. In conclusion, flowering significantly affects the production of seed set in *Anemone nemorosa*.

Introduction

Understanding the interactions between species is fundamental to understand the functioning of whole ecosystems. Climate change is one of the major factors affecting the species, interactions between species and ecosystems (Pachauri et al., 2014; Parmesan and Yohe, 2003; Hegland et al., 2009). One of the consequences of climate change is that it disrupts biological interactions between organisms by affecting the phenology of the organisms involved. However, different species respond differentially to climate change. (Miller-Rushing and Primarck, 2008; Roberts and al. 2015; Visser and Both, 2005).

Phenological data illustrate that most plant species in the temperate region are responding to global warming by flowering earlier (Fitter and Fitter, 2002). For example, Abu-Asab et al. (2001) showed that in some species the date of first flowering has advanced as much as 46 days over a 30 years period. Flowering earlier can have several costs on pollinator-plant interactions if there is a difference in the rate, or the magnitude of phenological change of the plant and its pollinators, i.e. if there is a phenological mismatch. Such a phenological mismatch can affect several different traits in both plants and pollinators, like seed set, species distribution, ecosystem functioning, and ecosystem services. The consequence of mismatches between mutualistic partners is not well studied, and for the moment the ecological consequences of climate-driven mismatches must be speculative.
Phenological mismatches between plant and pollinator may have several important consequences. Firstly, mismatches could diminish pollen disposition on the plant (Ashman et al. 2004). Moreover mismatches may reduce food availability for the pollinators. Although the effect of climate driven changes in food for pollinators, and pollinator availability for plants are, however, difficult to predict because our knowledge on the relative importance of bottom-up and top down forces in the population regulation is still poor (Steffan-Dewenter and Schiele, 2008). For example mismatches may result in cascading effects between the species (Waser and Real, 1979).

Pollinators are sensitive to climate variability (Morris et al. 2008), which may explain why the population dynamics of many pollinators vary strongly in time and space (Wiliams et al, 2001). In case of a specialist pollinator, a phenological shift will decrease its fitness (Memmott et al., 2007). In the boreal region, a shift of the flowering can have a dramatic impact because the period of food availability for pollinator is short (Kudo et al., 2008). A phenological mismatch between pollinator and plant may reduce plant seed set, which may have important long-term consequences for local persistence and dispersal of the species (Rafferty et al., 2015).

Most of the studies performed to investigate mismatches between plants and pollinators, have used crop species (Hovenden et al., 2008), and a very few have used wild plants (DeFrenne et al., 2010; Herrera, 1995; Lehtilä and Syrjänen, 1995; Rafferty and Ives, 2012). Most of the pollinators are insects, which are small and poikilothermic. Temperature is a critical factor affecting their life cycle development and activity pattern, which is particularly evident in alpine and artic regions. In the spring, a warmer climate will accelerate and modify the phenology.

In this study, I aiming to investigate primarily the impact of flowering times on seed set, proportion of self-pollination and diversity, availability, changing amount and diversity in spring and efficiency of pollinators.

I use a wild flower species *Anemone nemorosa*, and use variations in microclimate as a surrogate to study how species phenology might respond to climate change. Few studies (Andrea et al, 2008; De Frenne et al., 2010; Fitter and Fitter, 2002; Miller-Rushing et al., 2008) have shown the impact of the microclimate on the seed production and the pollinator diversity. The idea of this study is to simulate warm and cold conditions by using two different sites; north and south facing forest edges. A cold north site, with a late flowering time, represents a scenario where the pollinator phenology is more strongly affected by climate warming than flowering phenology. In contrast, a warmer site, with early flowering time, represents scenarios where flowering phenology is more strongly influenced by climate warming than pollinator phenology. My hypothesis is that under the colder climate the diversity and abundance of pollinators can be reduced due to comparatively less active pollinator as compared to warmer climate. However the pollinator can move over larger areas and hence their phenology may be less influenced by microclimate. I also suppose that pollinators do not have the same efficacy along the flowering times because the community of pollinators evolves over the time, especially in spring. Moreover I expect that composition and amount of pollinators will affect the seed production.

Materials and methods

Study species

Anemone nemorosa is a perennial plant and belongs to the family Ranunculaceae. The plant is found in Europe from northern Spain to northern Scandinavia and from Ireland to western Russia (Hultén and Fries, 1986). To our knowledge only one complete ecological study has been accomplished and is from the UK (Shirreffs, 1985). In general, the plant develops in deciduous or in mixed forest and can grow in a wide range of non-saline pH values (Shirreffs, 1985). It grows across a full range of soil texture like from sand to clay, relatively dry to wet soils and can sustain in open area like grassland (Shirreffs, 1985). The individual plant has a simple structure. The flower is held erect during the day, but closes and droops at night or during bad meteorological conditions (Shirreffs, 1985). The pollens from the flowers are spread by various flies, beetles, short tongued bees, syrphids and bumblebees (Shirreffs, 1985). Studies have been performed to investigate the reproduction through self-pollination, and concludes that self-pollination is very rare (Müller et al., 2000) and vegetative spread through rhizomes is more common (Brunet and Von Oheimb, 1998; Philip and Petersen, 2007; Shirreffs, D.A. and Bell 1984). This type of proliferation implies that *Anemone nemorosa* has the ability to generate a patch of many flowers, and that all flowers produced in the patch belong to the same genetically individual. Nevertheless, sexual reproduction by self-pollination or insect pollination, is the most important way for population persistence and spread (Brunet and Von Oheimb, 2008; Müller et al., 2000). The fruits (10-30 per flower) contains a single seed and are mainly gravity-dispersed, but according to the study from Delatte and Chabrerie (2008) myrmechory also occurs sometimes. A recent study showed that, *A. nemorosa* has the ability to modify its phenology (Roberts et al., 2015) by modify it chilling time. However, the deficient in knowledge is a major problem and most of the research on this species has been performed in UK or central Europe, and few studies have been made in Scandinavia. Also it is not known how the composition of pollinators varies over time during the flowering period in Scandinavia. Flowering period for *A. nemorosa* in Uppsala, Sweden according to www.artportalen.se (data from 2000 - 2014) is from end of March to mid of May and a peak of flowering in middle of April.

Figure 1: ***Anemone nemorosa (07/05/15)***

Study sites and experimental design

To test my hypotheses, I selected ten pairs (Figure 4) of sites around Uppsala. Each pair consisted of one north site and one south facing site display in figure 3. To assess whether pollinator abundance and composition differed I made two flowering times of observational studies. One were done in April (16^{th}-23th) and a second in early May (6^{th}-11^{th}).

Sites for the experiment were found by using Google Earth, in the west and south west of Uppsala city in Sweden. Ten pair of sites (picture 4) were selected, the selected sites consisted of mature and mixed deciduous and coniferous boreal forest, to ensure similar environmental condition in all the sites (humidity, light availability). The sites within a pair were at least 20m apart from the forest edge to avoid the edge effect. The minimal distances between to sites were at least 100m.

Treatments

Three treatments were applied; self-pollination, hand-pollination and wild-pollination. In each site, ten flowers were subject to each treatment.

- *Self-Pollination***:** the flower was completely covered (Fig. 2) before the flowering time by a fine-meshed nylon fabric during all the period to prevent access for pollinators, ants and gastropods (Türke et al., 2012). Since *A. nemorosa* practice mostly sexual reproduction (self-pollination is very rare according to Shirreffs, 1985), I suppose that plants exposed to this treatment will produce a minimal amount of seeds.
- *Hand-pollination*: by using a q-tip or another flower, the observer execute hand-pollination. After pollination the flower was immediately shielded by a fine meshed nylon bag (Fig. 2). By this treatment I estimate the maximal seed production, i.e. seed set will not be pollen limited.

- *Wild-pollination*: after observations and counting the number of insects pollinating the flower, the manipulator blocked the accessibility to flower by using a fine meshed nylon.

By using these three treatments, I want to test under what conditions insect pollination are important and how its effect may change over the flowering times and depend on warm and cold conditions

Figure 2: *Flower cover by a fine-meshed nylon.*

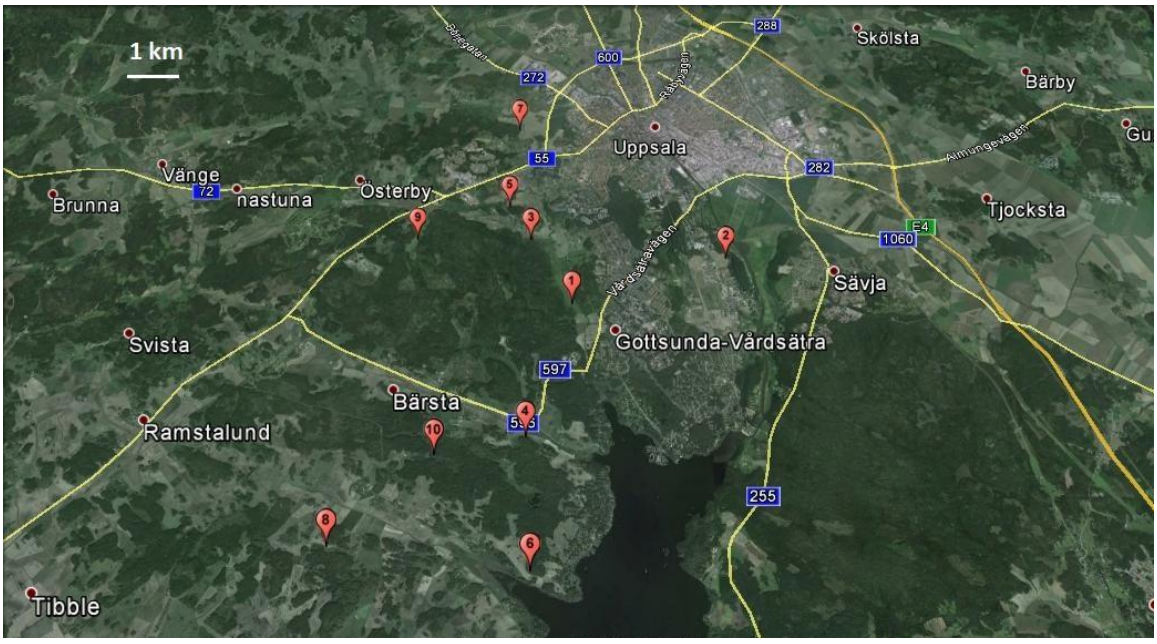

Figure 3: *Pair of sites location (Google Earth).*

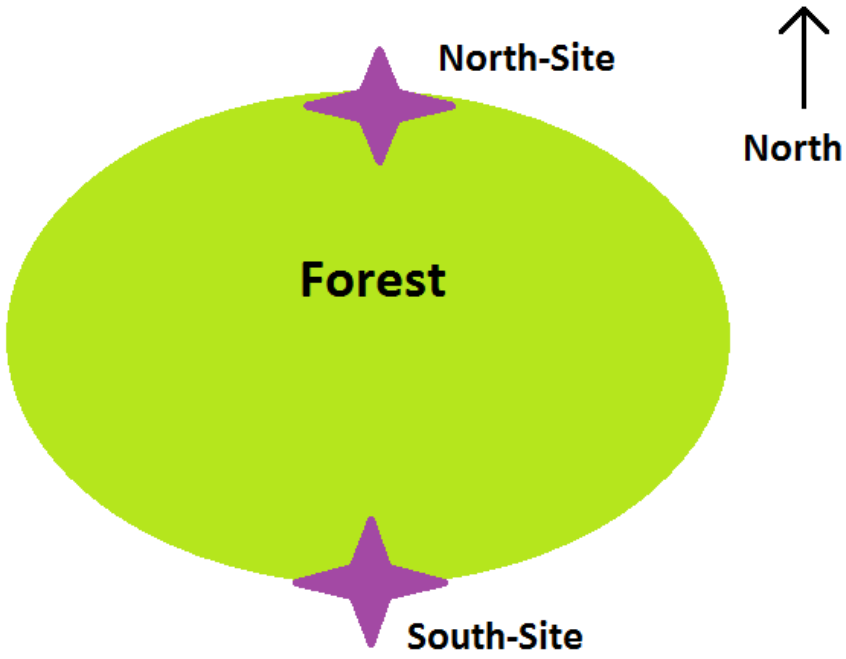

Figure 4*: Schema of one pair of site*

Field observations

In parallel of my treatments (self-pollination, hand-pollination, wild-pollination), I accomplished observations for 15 minutes per site (annex 1). The observation was performed during calm (no or low wind speed) and sunny days with a minimum temperature of 10° minimum. The observations included looking at several flowers, counting the number of visits for 15 minutes and recording the species or the family of pollinators. Bumblebees and butterflies were identified to the species level. Other insects, which could not be identified to the specie level in the field, were identified to the family level (for example Diptera, Heteroptera). The pollinators observed during the observational part of the study included *Bumbus terrestris* and *Bumbus lapidarius* (two species of bumblee bee) belonging to Apidea family and *Aglais urticae, Gonepteryx rhamni* and *Pieris rapae* which are butterfly.

Seed counting

One month after the different treatments were applied, the flowers were harvested. For the first flowering time around 600 flowers were selected, but only 398 flowers were harvested, as the remaining flowers were either rotten, missing, or lost (to herbivory or broken by wind and rainfall). The second flowering time consisted of around 500 flowers, and 321 flowers turned out as usable for harvest (annex 8).
All the harvested flowers were dried in the oven for 24h at 40°C. Then, the flowers were opened; seeds were counted. Two types of seed were identified: fertilized seed and unfertilized (Fig. 5). The fertilized are characterized by a green color and a size around 0.5cm – 1cm, the unfertilized is smaller (0.1-0.2cm) than the fertilized seed and have a pale color.

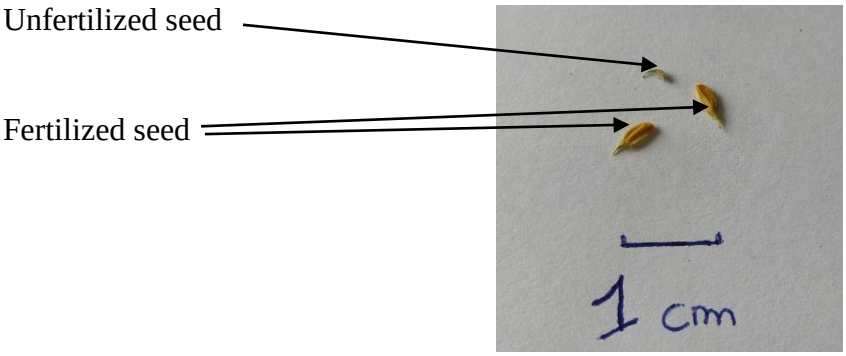

Figure 5: *Seeds fertilized (0.5cm) and unfertilized (0.1cm) of Anemone nemorosa*

Data analysis

Differences in fertilized seed production per flower among the different treatments were studied by analysis of the variance (ANOVA). Before conducting analyzes, I checked that the assumption for analysis of variance (homogeneity of the variance, residuals) were fulfilled. The distribution of seed numbers does not follow a normal distribution, but after inspection of the residuals we decided that analysis of the variance with a normal error distribution still would be ok. The ANOVAs were performed to investigate the effect of the factors and also for investigate if interactions exist (orientation / flowering time). I assess a difference among treatments as significant when P-values where 0.05 or lower.

To inspect the proportion of the seed that were fertilized (Fig. 6), I calculated the ratio between unfertilized and fertilized seeds.

To assess the pollination limitation (Fig. 7a), I used the number of seeds fertilized per flower. By site, I calculated the ratio between fertilized seeds from the treatment hand-pollination (means) and fertilized seeds from wild-pollination (mean). To test for effects of the different treatments I ran ANOVA with the ratio as response variable and the flowering time and treatments as dependent factors. Prior to the analyses, residuals were checked so that the underlying assumptions of ANOVA were fulfilled. .

To examine the relation between abundance diversity of pollinator and seed production, a linear model was been used (Fig. 9)
I tested the effects of the treatments on the proportion of fertilized seeds and unfertilized seeds by applying a generalized linear mix model with binomial error distribution.

To describe the composition of the pollinator community I calculated a Shannon Index (C.E. Shannon, 1948), by applying following formula:

$$H' = -\sum_{i=1}^{S} p_i \log_2 p_i$$

Where *Pi* is the proportion of individuals belonging to the *i* the species.

Pollination limitation was estimated according to the following formula:

Pollination limitation = (Fertilized seeds from wild-pollination treatment) / (fertilized seeds from hand pollination).

To test for differences between treatments I ran ANOVA after checking the residual and the homoscedasticity and the distribution (annex 9). The distribution differed substantially from a normal distribution, but due to a lack of data (N=35), a normal distribution do not fit accurately to my data. Moreover, I check the residuals and the homoscedasticity, my conclusion is an ANOVA can be done on this data.

Results

Effect of the pollinators on the seed production:

To investigate the effect of pollinators on seed production, I counted the proportion of fertilized seed for each treatment. Firstly, I observe that the median for the hand-pollination (17 seeds) and wild-pollination (17 seeds) treatments are similar. The means for self-pollination and hand-pollination are 3.07 and 19.19 fertilized seeds per flower, respectively, whereas the mean for wild-pollination is 16.72. (Fig. 6a) The hand-pollination and wild-pollination treatments significantly affect the number of fertilized seeds production when compared to self-pollination. However the difference between the hand-pollination and wild-pollination treatments is not significant (Annex 2).

(a) (b)

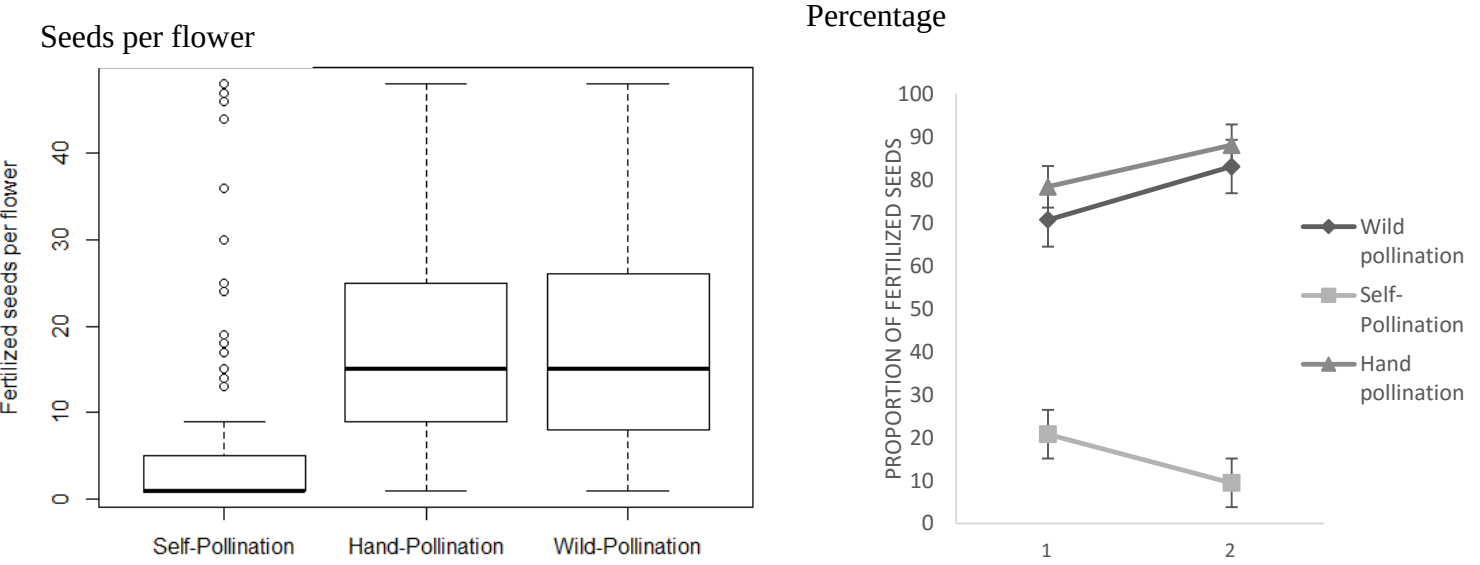

Figure 6: *Effects of the pollinator on the fertilized seed production (a) and the proportion of fertilized seeds during the two flowering times (1; Early Spring; 2: Late Spring) (b) (N self-pollination= 170, N hand-pollination = 273; N wild-pollination = 276)*

For the proportion of fertilized seeds (Fig. 6b), I observe for the two flowering times that hand-pollination have the highest ratio (Early spring = 79%; Late spring = 88%).Moreover, the ratio for the hand-pollination is not significantly different from that of wild-pollination. If I compare the self-pollination treatment and the wild-pollination treatment (Fig. 6b), I notice that the pollinator contribute a lot to the pollination of the seeds during the both periods (early and late spring).

However, my results show that there is no significant difference between hand-pollination treatment and wild-pollination treatment. About the self-pollination treatment, I found that the proportion decrease during the spring (Early Spring = 20%; Late Spring = 10%), I noticed that there have significantly difference between early spring and late spring (P =0.33: 4.04×10^{-7}, N=170, DF=1).

Effect of the microclimates and flowering times on the seed production

The pollination limitation per orientation and by time period is presented in Figure 7. The average between the two time periods differ (early spring = 0.7 and late spring=1). However the effect of the orientation (estimate = 0.17, P-value=0.44, Df=31, N=3) and the flowering times (estimate = 0.15, P-value =0.52, Df=31, N=35) are not significant. The distribution is a bit different between the groups, two groups have a large distribution (in early spring in the north and in the late spring in the south.

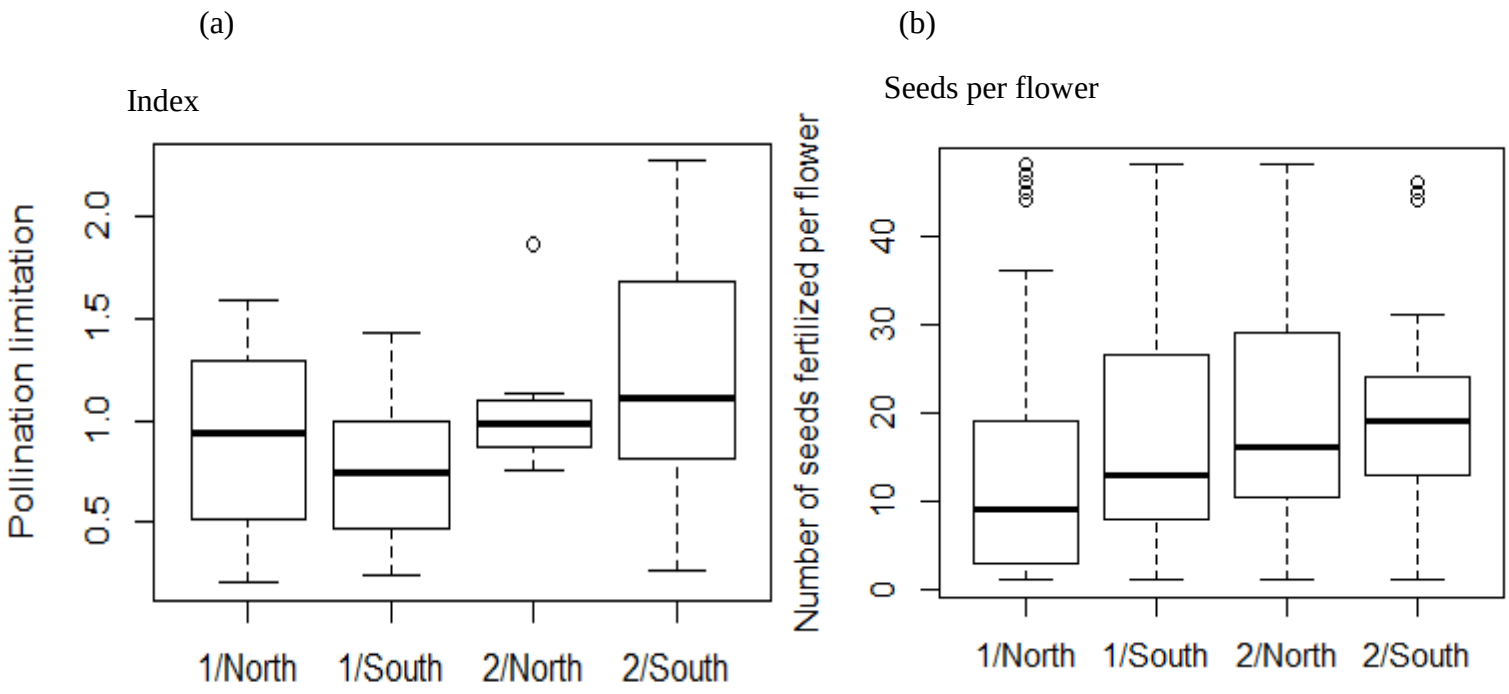

Figure 7: *Effect of the microclimates (North; South) and flowering time (1: Early Spring; 2: Late Spring) on the pollination limitation (Pollination limitation = (Fertilized seeds from wild-pollination treatment) / (Fertilized seeds from hand pollination) (a) and on the fertilized seeds production (b).*

The effect of flowering times on the number of fertilized seed production was significant (estimate = 5.31, P =0.0186, Df=285, N=289). However the orientation did not affect the fertilization significantly (estimate = 3.44, P = 0.12, Df=285, N= 289). Also there was no interaction between orientation of the sites and flowering times was observed (annex 5). During the early spring, the proportion of unfertilized seeds was higher at the north than the south site. At the south site, 50% of the flowers had 5 - 15 fertilized seeds per flower in the north site the results are similar around 50% of the flowers had 5 – 15 fertilized seed per flower. In the late spring, at the north site 25% flowers produced 10 - 15 fertilized seeds per flower whereas at the south site the most of flowers have a number of fertilized seeds ranging from 10 - 20 seeds.

Patterns in pollinator diversity and abundance: effects of orientation and flowering times.

There was no difference in pollinator abundance between the two flowering times, (P-value = 0.67, N=131, Df=14). There was no difference between the north and south face in neither the early (P-value = 0.31, N=57,Df=14) or in the late flowering times (P-value=0.38, N=74, Df=14).
The total number of insect-pollinators during early flowering times (57) was slightly lower than that of late flowering times (74). However the population of bumble bee was similar during both early and late flowering times (early flowering times: 19 ; late flowering times: 20), whereas population of butterfly was almost doubled during late flowering times (early flowering times: 7 and late flowering times:15). (Table 1)

Flowering time	Mid-April		Early-May	
	North	South	North	South
Bumbus terrestris	*5*	*14*	*10*	*10*
Bumbus lapidarius	*0*	*0*	*1*	*2*
Apis melifera	*0*	*1*	*5*	*6*
Aglais urticae	*0*	*1*	*1*	*3*
Gonepteryx rhamni	*1*	*5*	*0*	*9*
Pieris rapae	*0*	*0*	*0*	*2*
Diptera	*10*	*19*	*11*	*14*
Heteroptera	*0*	*1*	*0*	*0*
Total	***16***	***41***	***28***	***46***

Table 1 : *Pollinator observation (individuals), the observations were done at two flowering times (Mid-April ; Early-May) N=131.*

Pollinator abundance :
The pollinator abundance was significantly affected by the microclimate (estimate = 1.30, P-value=0.0138, Df=36, N=40) and was higher for south site than the north site. Whereas, the flowering times had no significant effect on pollinator abundance (estimate: 0.9, P-value=0.082, Df=36, N=40).

Pollinator diversity **:**
I calculated the shannon index during both early and late spring for both north and south sites to show the pollinator diversity. The flowering times had no significate impact (estimate=1.9, P-value=0.239, Df=36, N=40) on the pollinator diversity. However the microclimate significantly affected pollinator diversity (estimate = 3.80, P-value=0.0221, Df=36, N=40). No interaction between both of these factors was recorded (annex 6). However I noticed that during both flowering times at south site the distribution was larger than at north site throughout the flowering times.

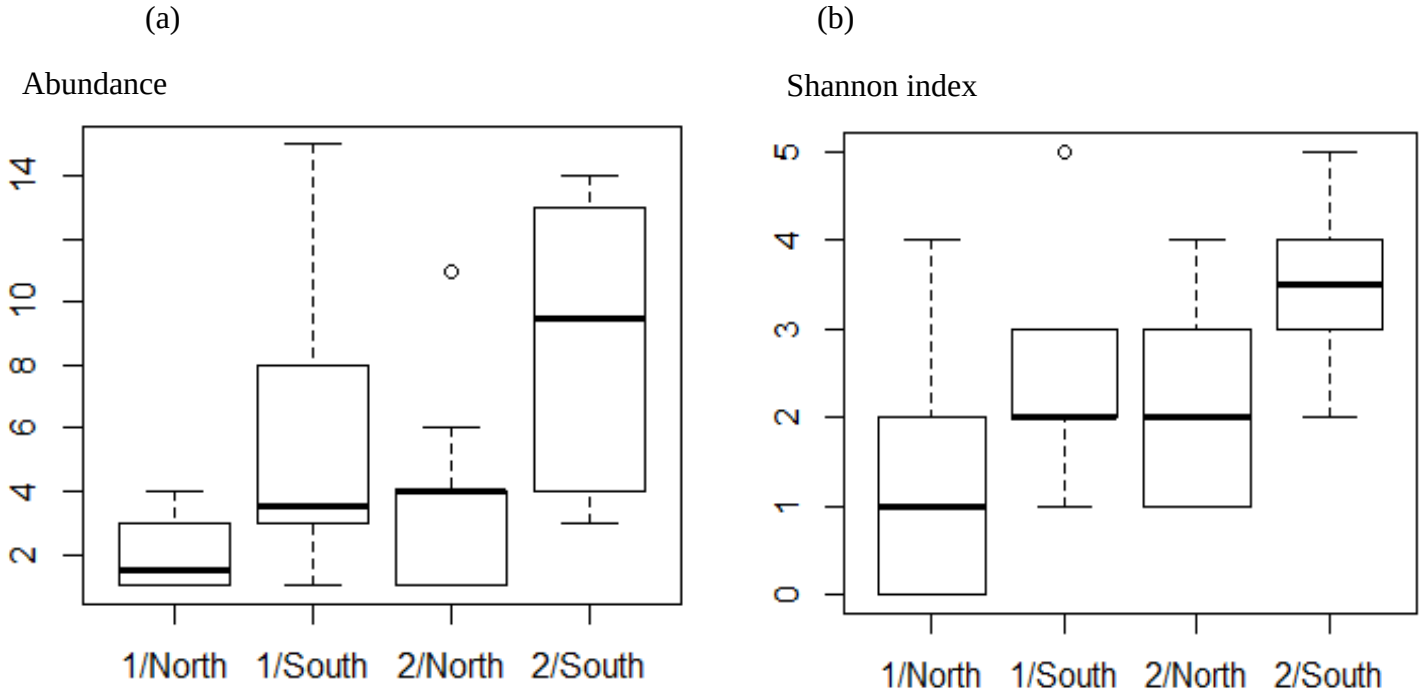

Figure 8: *Effect of the microclimates (North; South) and the flowering times (Early Spring; Late Spring) on the pollinator abundance (a) and on the pollinator diversity (Shannon index) (b).*

Relating fertilized seed set to pollinator diversity and abundance.
I found no correlation between the seed production and the diversity or abundance of pollinators. I get the same results if I test with the ratio (total seeds / fertilized seeds). Also, by looking on the table 1, I observe that the main pollinators are bumble bees (*Bombus terrestris* and *Bombus lapidarius*) and Dipteras. Other investigations by test only the main pollinators (*Bombus terrestris* and Dipteras) have been done, but all of this tests found no relation between abundance or diversity and the seed set.

(a) (b)

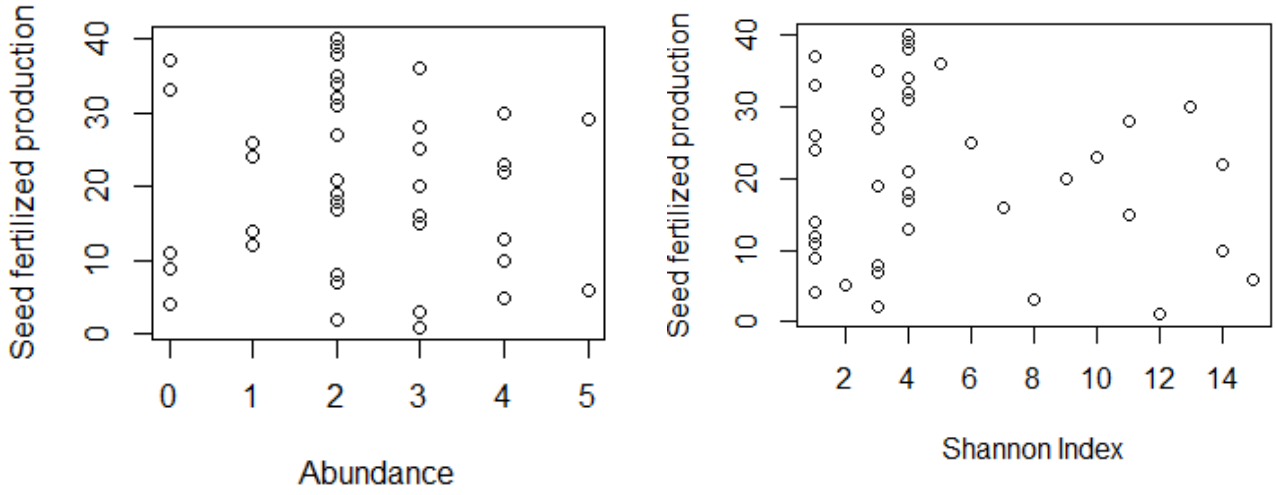

Figure 9: *Linear model of seed fertilized relating to the pollinator abundance (a) and the Shannon index (b). See table 1 for the composition of pollinator.*

Discussion:

This study reveals that there is correlation between the flowering times and seed set, with higher proportion of fertilized seeds in late than early spring. The data show that there is no significant shift of in the diversity and abundance of pollinator over time. The microclimate also has no significant effect on the abundance and the diversity. The experimental sites were placed on the south and the north, my hypothesis was that the south facing sites would produce less fertilized seeds than north-facing sites due to higher density of pollinators and a more divers pollinator community, but I found no support for this hypothesis.

Pollination limitation and self-pollination

The hypothesis was that self-pollination treatment should result in the lowest seed production (Shirreffs 1985). For the hand-pollination treatment, I assumed that I would remove any potential pollen limitation and thus expect the maximal seed production. The wild-pollination should, depending on the degree of pollinator limitation, have a seed production in-between the self-pollination and the hand-pollination treatments. Based on my results I can confirm my hypotheses that seed production is limited if only self-pollination is allowed. I found lower production of seeds for the self-pollination treatment, in compare to hand-pollination and wild-pollination treatment. My experiments showed that there is no difference between the hand and the wild-pollination at any time and under both microclimates. So we can interpret that the pollinator is efficient enough to fulfill the plant's demand of pollen. For *A. nemorosa* it appears that only a few visits are required to meet the pollen demand of the plant and to ensure high seed set.

Anemone nemorosa should produce self-incompatible pollens (Shirreffs 1985), in this study the self-pollination treatment show that it is not the totally true. Self-pollination is very common in plants and one of the criteria why I choose *A. nemorosa*, is because this specie do not produce a large amount of seed induced by self-pollination. That is why it is easier to quantify and investigate the effect of the

phenology and the microclimate. Self-pollination facility the transmission, and promote a reproductive assurance for the species. However many studies show that the self-pollination generate an increasing of the inbreeding depression due to the fact that the individuals have low genetic diversity (Charlestworth et al., 2003). Inbreeding increases the level of homozygote and generally leads to a decreased of the fitness for example inbreeding increase the pollen discounting. (Charlestworth et al., 2003). For avoid self-pollination, plants develop many different strategies such as temporal and physiological isolation (Charlestworth et al., 2003).

In this study, I recorded that the self-pollinated flowers produce seeds. One reason could be that before I enclosed the flower with the bag, the flowers were open for some time and pollinators have had the opportunity to visit the flowers, which is very difficult to avoid. Or the self-pollination for *Anemone nemorosa* represent a "safe way" in case of a lack of pollinator and then ensure the species continuity, especially in early spring when the pollinator's abundance can be low. A study on *Clarkia xantiana* found a relationship between the amount of pollinator and the rate of self-pollination (Fausto et al., 2001). However some species such as *Helleborus foetidus* respond differently in absence or a lack of pollinator (Herrera et al., 2001). Shirreffs (1985) found that self-pollination respond against a low abundance of pollinator and the proportion of the self-reproduction was not quantified. Another explanation is that low levels of self-fertility observed in previous studies could be explained by geographical variation. Moreover, it could be interesting to reproduce this type of experiment along a geographical gradient, e.g. from southern to northern Sweden, and examine if the self-pollination increase towards the north for confirm that last hypothesis.

What the effect of the flowering time and microclimate on the seed set and relationship with pollinator population?

The flowering time effect on the production of fertilized seeds was complicated and significantly less production of fertilized seeds was observed in mid-April as compared to early May. De Frenne et al. (2010) also found positive relationship between cumulative temperature during the winter and seed set. Although this difference in fertilize seed set between the two flowering times is around 10%. Rafferty and Ives (2012) found that the pollinators do not have the same effectiveness during the spring and they found that the changing pollinator assemblage during the two flowering times had significant effect on the fitness of the species. However the pollination limitation in my study show that the pollinators have the same efficiency at both flowering time.

The community of pollinator has the same abundance and diversity at both flowering times. Moreover the microclimate significantly affects the diversity and the abundance of the pollinator. In early spring, I recorded fewer butterflies and common bees during the first flowering time in comparison with second flowering time. Common bees were more common during the second flowering time and it is probably due to the presence of bee hives near to some sites. Also most of the butterflies emerge in mid of April. One hypothesis could be that the cold microclimate can reduce the mobility of the pollinators and the pollinators prefer to go to warmer place due to their physiology. Moreover, no significant relationship between the pollinator and the seed set maybe due to insufficient data.

The hand-pollination treatment and the individual concept?

As, *A. nemorosa* has the ability to produce many clones to conquer the new environment. That is why, most of the time A. *nemorosa* can develop a large rhizome on the ground by asexual reproduction (Shirreffs 1985). It is therefore common to observe a patchy distribution of this species in a forest. As a result, when I did the hand pollination some of the pollen that I transfer pollen from one flower into another flower, may have belonged to the same genetically individual. Theoretically, the results of the self-pollination and the cross pollination, should be identically, because in both case it will be the same individual. However the results show that treatments are different. So probably that most of the case I cross two different individuals or more, and few cross within the same individual occurred.

Results distorted by the weather?

Abnormal warm spring during my experiment probably skew my results. Although the snow melted early around mid-February, the spring was colder than the usual (annex10). I observed that the mean temperature during this year was 6.63°C in April and 9.41°C in May. The long term mean record (1961-1990) show that in April is 4°C and 10.2°C in May. In general according to the record from January to April the temperature mean were higher in comparison to the previous years. This cumulative temperature could also explain the distorted results. De Frenne *et. al.* found that the cumulative temperature in the winter and early spring is a critical factor for the fitness of the early species such as *A. nemorosa.*

Could other factors explain these results?

My experiment was conducted in a natural environment, then, it was not possible for me to measure and quantify all the different factors which can interact with the flowering time and the seed set.
Few abiotic factors are known to affect the flowering time, such as temperature or the brightness level. For instance, warm temperature would promote an early flowering time and increase the number seeds. Also the soil composition have not been recorded, and it is not known that the soil play and important role on the physiology and on the phenology of the plant. The soil has different property such as the granulometry, density, chemical compounds, all of this factors have significant effect on the water storage, the nutrient availability.
Biotic factors, such as interspecific competition among the flowers to attract the pollinators have not been recorded. For example, I could incorrectly assume that a large population of flowers attracts more pollinators than a small population. However, it is likely that the pollinators will not pollinate only one plant species, especially if others have physiological advantages, like longer stalk or a higher volume of nectar.

How is A. nemorosa affected by a global warming phenological mismatch with its pollinators?

With the results of my experiment and the literature, I can speculate that the global warming might have an effect on *A nemorosa* fitness, because the results showed that this species produce more fertile seeds if it warmer because April was a bit colder than May. I showed type of plasticity because the self-pollination variate throughout the spring. Moreover my results demonstrate that high seed set can be achieved already during the early spring and that the pollinator present during that time period appears

to be able to fulfill the pollination that the plant need to maximize its seed set. However I have only studied pollination and seed set, and to fully understand its response to climate change the response of other traits need to be addressed before conclusions can be made.

Conclusions and Future work

Anemone nemorosa is a good species model to investigate the impact of flowering time during spring. In this study, I found that different treatments have different effects on the seed set. However, I was not able to identify variation on the abundance or diversity of the pollinator population over time. Thus, it is likely that there are enough pollinators present that can fulfill the plants' pollen demand. My experiment did not show an effect of the microclimate on the fertilized seed production. However, I detected variation on the rate of self-pollination throughout the spring, which might be a strategy used by the species to ensure the next generation offspring. Future experiments should include the measurement of biotic and abiotic factors, such as flower competition, soil composition, and humidity, which were not considered in this study, but may play a critical role in the flowering time.

To investigate further the effect of microclimate and the pollinators on the reproduction of *A. nemorosa* more data is required. Future work should also include more observations sites to increase the power of samples. Moreover, try to germinate the seeds after harvested, could be a good fitness proxy. Also the design of the study should be revisited to include the monitoring of other factors regarding microclimate such as temperature, humidity, soil, etc. Also the longer observation time will give better idea about the pollinator population, diversity and abundance and their correlation with fertilized seed set. Also by recording severals different factors, it is possible to compute a niche modeling and try to find a correlation among the factors, and determine which factors affect more the flowering time and the seed set.

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Annex

		Early Spring		*Late Spring*	
		North	South	North	South
Site 1	Self-pollination	21/04	21/04	05/05	05/05
	Hand-pollination	21/04	21/04	05/05	05/05
	Wild-Pollination	21/04	21/04	05/05	05/05
	Observation	21/04	21/04	05/05	05/05
Site 2	Self-pollination	18/04	18/04	08/05	08/05
	Hand-pollination	21/04	21/04	08/05	08/05
	Wild-Pollination	21/04	21/04	08/05	08/05
	Observation	21/04	21/04	08/05	08/05
Site 3	Self-pollination	20/04	18/04	11/05	11/05
	Hand-pollination	20/04	20/04	05/05	05/05
	Wild-Pollination	23/04	20/04	11/05	11/05
	Observation	23/04	23/04	11/05	11/05
Site 4	Self-pollination	15/04	15/04	07/05	07/05
	Hand-pollination	18/04	18/04	07/05	07/05
	Wild-Pollination	22/04	22/04	07/05	07/05
	Observation	22/04	22/04	07/05	07/05
Site 5	Self-pollination	20/04	20/04	06/05	06/05
	Hand-pollination	20/04	20/04	06/05	06/05
	Wild-Pollination	23/04	23/04	06/05	06/05
	Observation	21/04	21/04	06/05	06/05
Site 6	Self-pollination	15/04	15/04	07/05	07/05
	Hand-pollination	18/04	18/04	07/05	07/05
	Wild-Pollination	22/04	22/04	07/05	07/05
	Observation	22/04	22/04	07/05	07/05
Site 7	Self-pollination	18/04	18/04	06/05	06/05
	Hand-pollination	20/04	20/04	06/05	06/05
	Wild-Pollination	23/04	23/04	06/05	06/05
	Observation	23/04	23/04	06/05	06/05
Site 8	Self-pollination	16/04	16/04	07/05	07/05
	Hand-pollination	22/04	22/04	07/05	07/05
	Wild-Pollination	22/04	22/04	07/05	07/05
	Observation	22/04	22/04	07/05	07/05
Site 9	Self-pollination	16/04	16/04	06/05	06/05
	Hand-pollination	20/04	22/04	06/05	06/05
	Wild-Pollination	20/04	22/04	06/05	06/05
	Observation	22/04	22/04	06/05	06/05

Site 10	Self-pollination	16/04	16/04	07/05	07/05
	Hand-pollination	22/04	22/04	07/05	07/05
	Wild-Pollination	22/04	22/04	07/05	07/05
	Observation	22/04	22/04	07/05	07/05

Annex 1: Date of establishment treatments and observations (15min by site)

```
lm(formula = Nb.seed.fertilized ~ Treatment)

Residuals:
   Min     1Q  Median     3Q    Max
-17.325  -8.325  -5.007   5.993  39.724

Coefficients:
                                Estimate Std. Error t value Pr(>|t|)
(Intercept)                        8.276      1.044   7.924 8.36e-15 ***
Treatment Hand-pollination        10.049      1.316   7.634 6.96e-14 ***
Treatment Wild pollination         9.730      1.316   7.392 3.88e-13 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 13.62 on 745 degrees of freedom
Multiple R-squared:  0.08517,           Adjusted R-squared:  0.08272
F-statistic: 34.68 on 2 and 745 DF,  p-value: 3.972e-15
```

Annex 2: Treatments effect on the seed set (all the flowering times / all the orientations) output from Anova.

```
Call:
lm(formula = as.numeric(Nb.seed.fertilized) ~ microclimate + as.factor(Flowering time),
    data = seedset[seedset$Treatment == unique(seedset$Treatment)[3],
        ])

Residuals:
   Min     1Q  Median     3Q    Max
-19.372  -9.372  -3.763   6.869  32.237

Coefficients:
                    Estimate Std. Error t value Pr(>|t|)
(Intercept)           15.763      1.417  11.124   <2e-16 ***
N.SSouth               1.367      1.617   0.846    0.399
as.factor(Flowering time)2    3.241      1.617   2.004    0.046 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
Residual standard error: 13.67 on 286 degrees of freedom
Multiple R-squared:  0.01527,           Adjusted R-squared:  0.008382
F-statistic: 2.217 on 2 and 286 DF,  p-value: 0.1108
```

Annex 3: Effect of microclimate and flowering times on the fertilized seeds production.

```
Call:
lm(formula = limitation ~ microclimate * flowering time)

Residuals:
    Min      1Q  Median      3Q     Max
-0.97652 -0.26373 -0.07096  0.32676  1.04085

Coefficients:
                    Estimate Std. Error t value Pr(>|t|)
(Intercept)           0.9157     0.1513   6.052 1.06e-06 ***
orientationS         -0.1698     0.2140  -0.793    0.434
flowering time2       0.1492     0.2270   0.657    0.516
orientationS:flowering time2   0.3385     0.3273   1.034    0.309
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.4785 on 31 degrees of freedom
Multiple R-squared:  0.1331,           Adjusted R-squared:  0.04916
F-statistic: 1.586 on 3 and 31 DF,  p-value: 0.2127
```

Annex 4: Effect of orientation and time on the insect pollination.

```
Call:
lm(formula = as.numeric(Nb.seed.fertilized) ~ microclimate * as.factor(Flowering time),
    data = seedset[seedset$Treatment == unique(seedset$Treatment)[3],
        ])

Residuals:
   Min      1Q  Median      3Q     Max
-18.963 -10.101  -3.115   7.037  33.348

Coefficients:
                                      Estimate Std. Error t value Pr(>|t|)
(Intercept)                             14.652      1.643   8.915   <2e-16 ***
N.SSouth                                 3.449      2.249   1.533   0.1263
as.factor(Flowering time)2               5.310      2.243   2.368   0.0186 *
N.SSouth:as.factor(Flowering time)2     -4.297      3.232  -1.330   0.1847
```

```
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 13.65 on 285 degrees of freedom
Multiple R-squared:  0.02134,           Adjusted R-squared:  0.01104
F-statistic: 2.071 on 3 and 285 DF,  p-value: 0.1042
```

Annex 5: Fertilized seeds production and effects of orientation, flowering times and their interaction.

```
Call:
lm(formula = shannon ~ microclimate * flowering time)

Residuals:
   Min     1Q Median     3Q    Max
 -5.7   -2.9    0.0    1.9    9.1

Coefficients:
                    Estimate Std. Error t value Pr(>|t|)
(Intercept)                  2.100      1.123   1.870   0.0696 .
OrientationS                 3.800      1.588   2.393   0.0221 *
flowering time2              1.900      1.588   1.196   0.2393
OrientationS:flowering t     ime2    0.900      2.246   0.401   0.6910
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 3.551 on 36 degrees of freedom
Multiple R-squared:  0.3439,           Adjusted R-squared:  0.2892
F-statistic: 6.289 on 3 and 36 DF,  p-value: 0.001526
```

Annex 6: Flowering times, orientation effect and their interaction on the Shannon index.

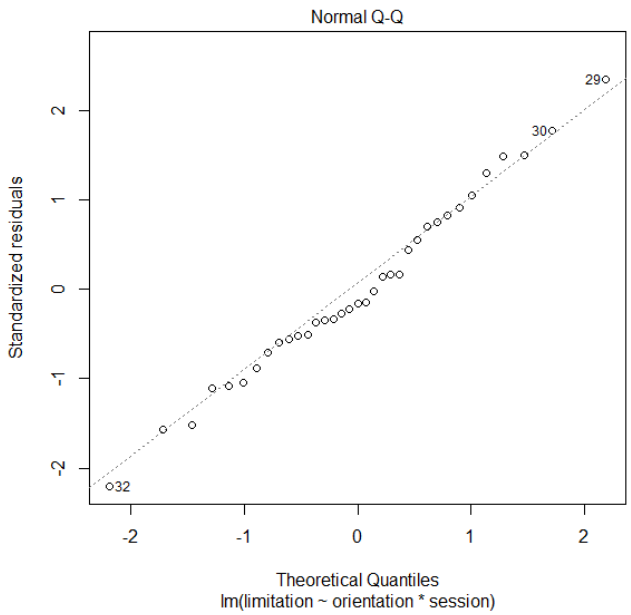
```
Call:
lm(formula = abundance ~ microclimate * flowering time)

Residuals:
   Min     1Q Median     3Q    Max
 -1.50  -1.10  -0.10   0.65   2.80

Coefficients:
                    Estimate Std. Error t value Pr(>|t|)
(Intercept)          2.200e+00  3.551e-01   6.195 3.82e-07 ***
OrientationS          1.300e+00  5.022e-01   2.589   0.0138 *
flowering time2              9.000e-01  5.022e-01   1.792   0.0815 .
OrientationS:flowering time2 -4.213e-16  7.102e-01   0.000   1.0000
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

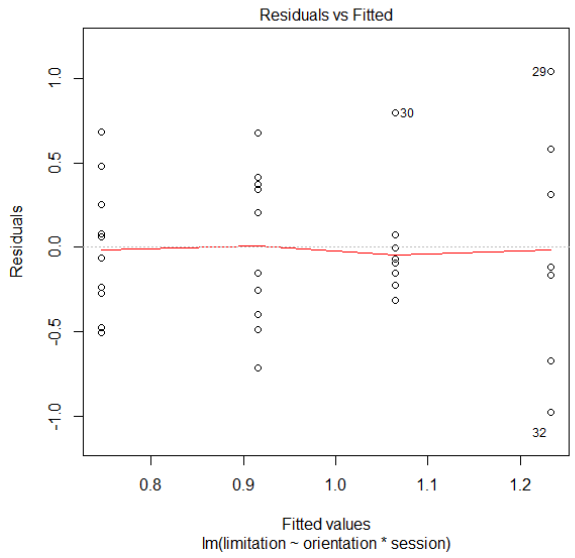
Residual standard error: 1.123 on 36 degrees of freedom
Multiple R-squared: 0.3551, Adjusted R-squared: 0.3014
F-statistic: 6.608 on 3 and 36 DF, p-value: 0.001133

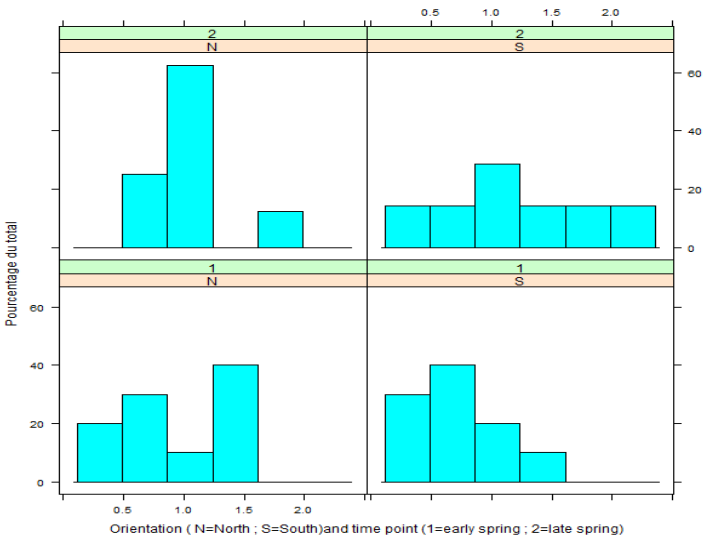
Annex 7: Time, orientation effects and their interaction on the pollinator abundance.

	Early Spring	Late Spring	Total
Self-Pollination	122	48	170
Hand pollination	128	145	273
Wild pollination	148	128	276
Total	*398*	*321*	

Annex 8: Flowers usable per treatment and time.







Annex 9: Distribution, residual and homoscedasticity (pollination limitation)

Day	January	February	March	April	May
Mean (T°C)	0.2	0.7	3.2	6,6	9,4
Long-term mean (1961-1990)	-4.4	-4.6	-1	4	10,2

Annex 10: Temperature in Celsius in Ultuna (Uppsala, Sweden) from January to May 2015