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Host plant preference of *Phyllotreta nemorum*

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Contents:

Summary

Flea beetles (*Phyllotreta nemorum*) are small beetles, which live and feed on a number of (mostly cruciferous) host plants. Some of the flea beetles carry R-genes which enable them to feed on a specific host plant, the G-type of *Barbarea vulgaris* ssp. *arcuata*, while the others can not. Two locations have been found where resistant flea beetles are living on this G-type of *B. vulgaris* ssp. *arcuata*. Since the R-genes allow the flea beetles to live and feed on an additional host plant it is to be expected that these R-genes spread rapidly through the populations. Most populations however contain only few resistant individuals. Limited migration may be one of the factors influencing the spread of R-genes. One of the possibilities is that host plant preference is (partly) responsible for this. If resistant flea beetles prefer living on *B. vulgaris* ssp. *arcuata* (G-type), while susceptible flea beetles prefer living on another host plant this will influence the gene flow. Host plant selection can be divided in host plant finding and host plant acceptation. This report focuses mainly on preference based on olfactory cues, which are used preliminarily in host plant finding. In our experiments the flea beetles were not allowed to touch the leaves. In the experiment two plants were used, *Raphanus sativus* and *B. vulgaris* ssp. *arcuata* (G-type). Also a green paper fake leaf was used. The research questions for the experiment were: Does *P. nemorum* prefer some host plants over others? Is there any difference in preference between individuals which are susceptible and resistant to the defences of *B. vulgaris* ssp. *arcuata* (G-type)? Do resistant *P. nemorum* have a preference for *B. vulgaris* ssp. *arcuata* (G-type) as a host plant? Do susceptible *P. nemorum* have a preference for *R. sativus* over *B. vulgaris* ssp. *arcuata* (G-type)? Does host plant preference influence the population structure of *P. nemorum*?
To answer these questions olfactometer tests were done with leaves of *B. vulgaris* ssp. *arcuata* (G-type) vs *R. sativus*, *B. vulgaris* ssp. *arcuata* (G-type) vs an empty part of the olfactometer, *R. sativus* vs an empty part and a green paper leaf vs an empty part. These tests were performed with resistant flea beetles. With susceptible flea beetles, only olfactometer tests were done with *B. vulgaris* ssp. *arcuata* (G-type) vs *R. sativus.* These tests showed that (resistant) flea beetles indeed prefer the side with a leaf over the side without a leaf, except for the paper leaf. In this test the flea beetles tended (not significantly) to go to the empty side. These tests imply that the flea beetles are able to detect the plants in the olfactometer. Since the flea beetles showed no preference for or tendency towards the paper leaf they detected the leaves by means of olfactory cues and not visual cues. In the test with the paper leaf vs an empty half the flea beetles jumped significantly more than in the other tests. This can be explained by the fact that the flea beetles can not detect any host plant in their surroundings. Jumping is for flea beetles besides walking the most important way of moving. The lack of a suitable host plant in the area makes it likely that the flea beetles were trying to get closer to a host plant by jumping. Both susceptible and resistant flea beetles tended to be slightly more on the side of *B. vulgaris* ssp. *arcuata* (G-type) than on the side of *R. sativus*, but this was not significant. Neither the susceptible nor the resistant flea beetles showed a clear preference for the olfactory cues of either *B. vulgaris* ssp. *arcuata* (G-type) or *R. sativus*. Therefore it seems to be unlikely that the population structure of the flea beetles and thus the spread of the R-genes is mainly influenced by host plant preference. More research has to be done, however, to substantiate this conclusion. The preference for a host plant might after all only become visible when the flea beetles get the chance to touch and taste the plant.

Introduction

The flea beetle, *Phyllotreta nemorum* L (Coleoptera: Chrysomelidae: Alticinae), is a small herbivore insect (De Jong et. al., 2001). They are called flea beetles because they can jump remarkably high for their size. The eggs hatch after a few days, then larvae emerge. These larvae are leaf miners (De Jong et. al., 2001): they live inside leaves of a few cruciferous plant species like *Sinapis arvensis, Raphanus sativus, Raphanus raphanistrum* and *Barbarea vulgaris* ssp. *arcuata* and in *Cardaria draba* (De Jong et. al., 2001; Nielsen and De Jong, 2005; De Jong and Nielsen, 2000; De Jong and Nielsen, 1999). *S. arvensis* is the host plant which is most often used by *P. nemorum* (Nielsen and De Jong, 2005). After a few weeks the adult flea beetles emerge. The larvae have lived through their larval stages, they left the leaves, they dug into the soil to pupate and climbed back up as adult. They can feed on the leaves of the same plant species as they did when they were larvae (De Jong et. al., 2001).

There are signs which indicate that *Barbarea vulgaris* (ssp. *arcuata*) is a relatively new host plant which was unsuitable for *P. nemorum* until recently (Nielsen and De Jong, 2005; Nielsen, 1997a; De Jong and Nielsen, 2000). Two different types of this plant exist, a glabrous (G-type) and a pubescent (P-type) form (Nielsen, 1997b). The P-type has dense simple hairs on the surfaces of rosette leaves (Agerbirk et. al., 2003). Only one of these two forms is suitable as a host plant for all *P. nemorum*, the P-type (De Jong et. al., 2001; De Jong and Nielsen, 2002). Although it is rarely used as a host plant in the field, this plant is unable to defend itself against *P. nemorum* (Nielsen and De Jong, 2005). The G-type however is able to defend itself against *P. nemorum* in the summer (De Jong and Nielsen, 2002). The plant has a type of defence which makes it impossible for most flea beetles to use it as a host plant. A few flea beetles however are able to use the G-type as a host plant (Nielsen, 1997a). They are resistant to the defences of the plant. In the rest of this report the G-type of *Barbarea vulgaris* (ssp. *arcuata*) is indicated with *B. vulgaris* unless stated otherwise. The resistance is regulated by R-genes (De Jong et. al., 2001; Nielsen and De Jong, 2005; De Jong and Nielsen, 2002). Resistance in the form of R-genes can be found on X-linked, Y-linked and autosomal genes (De Jong and Nielsen, 1999; Nielsen, 1997a; De Jong and Nielsen, 2000). Both heterozygotes and homozygotes for this R-gene are resistant to the defences of *B. vulgaris*, as it is a dominant gene.
Populations of *P. nemorum* living on *B. vulgaris* consist of 95-100 % resistant flea beetles, while populations living on other host plants contain only 7,0-71,4 % resistant flea beetles; even if these populations are less than one km away from an entirely resistant population (De Jong et. al. unpublished; Nielsen and De Jong, 2005).
The R-gene allows the flea beetles to feed on *B. vulgaris*: individuals with the R-gene have this advantage over individuals without the gene. This advantage makes it likely that the gene will spread rapidly through the populations. Research has however shown that this is not the case, especially if *B. vulgaris* is not around (De Jong et. al., unpublished; Nielsen and De Jong, 2005). Nielsen and De Jong (2005) recorded that the R-genes in *P. nemorum* populations living on *S. arvensis* became less frequent after a while. In a population feeding on *B. vulgaris* the R-genes had a more stable frequency. Also a previous allozyme study showed little difference in allozymes between different populations (Nielsen and De Jong, 2005; De Jong et. al., 2001). There are several ways to explain this. It is possible that there is a limited amount of gene flow between populations on different host plant species (Nielsen and De Jong, 2005). This could be because of limited migration between different host plants. Another possible reason for the limited spread is that there is a trade-off involved in the possession of R-genes (De Jong et. al., unpublished; De Jong and Nielsen, 2000). An experiment suggests that a co-adapted gene complex is involved (De Jong and Nielsen, 2002).

When a homozygote resistant individual is mated with a susceptible one, heterozygotes result. When the heterozygotes are mated with susceptibles for a couple of generations and then two heterozygotes from that rearing are crossed no homozygote resistant flea beetles occur. This suggests that the R-gene may need additional genes in order to let the homozygotes for this R-gene survive (De Jong and Nielsen, 2002).

Until now the only locations known where resistant *P. nemorum* populations live on *B. vulgaris* are in Ejby and in Kværkeby in Denmark (De Jong and Nielsen, 2000). Less than 20 % of the presently known locations containing *B. vulgaris* are home to *P. nemorum* (Nielsen and De Jong, 2005). These locations can be divided in different sublocations, since the areas are big and within one such area the host plants stand in patches, a number of plants closely together at some distance from another group of plants. In some cases these patches are further apart than in others. In both Ejby and Kværkeby not only *B. vulgaris* is present but also other host plants like *Sinapis arvensis* and *Brassica nigra*.
The distribution of the host plants suggests that *P. nemorum* might live in a metapopulation. This would mean that the amount of gene flow within patches is higher than the amount of gene flow between patches and that the amount of gene flow within one location is higher than between locations. This might explain why different levels of R-gene frequencies exist within one location. To migrate, *P. nemorum* will usually walk or jump although they can also fly (De Jong et. al., 2001; De Jong and Nielsen, 2002). Because walking and jumping are relatively slow ways to move, the migration between locations might be relatively small. This might also explain why there are only two locations found with resistant flea beetles. If the chance of a flea beetle reaching a new location is small, the chance of a resistant flea beetle to reach another location with *B. vulgaris* is even smaller.
It could even be the case that *P. nemorum* living on *B. vulgaris* have a genetic preference for this host plant; this could also explain why relatively low numbers of R-genes are found in populations close to a population living on *B. vulgaris*.

The selection of a host plant can be divided in finding the host plant and in accepting the host plant (Thorsteinson, 1960 according to Finch and Collier, 2000). Factors influencing host plant finding are olfactory cues and visual cues although it is uncertain how important both factors are (Finch and Collier, 2000). The visual cues can be green and the shape of the leaves or its shade, olfactory cues are volatiles emitted by the plant. Visual cues are thought to be important in landing of flying insects (Finch and Collier, 2000), while olfaction is thought to be a very important factor in host plant finding; for *Plutella xylostella* for example it is the most important factor (Couty et. al., 2006). Others doubt if olfactory cues are that important in finding a host plant since the volatiles are blown away with the wind quickly and are therefore only detectable from a relative small distance (Finch and Collier, 2000).
When a phytophagous insect finds the plant, it still has to decide whether it actually wants to feed on it or not. For this accepting of the host plant chemical substances on the leaves are important. Three stages have been found in prefeeding behaviour for some phytophagous insects like for example *Phyllotreta cruciferae*, an acclimation stage, a stimulation stage and initial feeding (Henderson et. al., 2004). In these stages the herbivorous insects touch the surface of the leaf with both their feet and their antennae (Henderson et. al., 2004). This touching enables them to detect the chemicals of the plant and thereby they can decide whether they will eat the plant or not. In determining the preference of an insect for a plant species over another both host plant finding and host plant acceptation may be important. Finding a host plant will be the first step, but if it does not accept it after that, the plant is still not preferred or used as a host plant more often.

The aim of this research was to determine whether it is possible that the population structure of *P. nemorum* is influenced by host plant preference. It is possible that the spread of R-genes through different populations is prohibited by the preference of resistant *P. nemorum* for *B. vulgaris* as a host plant.
The research questions were:

-Does *P. nemorum* prefer some host plants over others?

-Is there a difference in preference between individuals which are susceptible and resistant to the defences of *B. vulgaris*?

-Do resistant *P. nemorum* have a preference for *B. vulgaris* as a host plant?

-Do susceptible *P. nemorum* have a preference for *R. sativus* over *B. vulgaris*?

-Does host plant preference influence the population structure of *P. nemorum*?

The hypotheses were:

-*P. nemorum* has a preference for certain host plants over others.

-The susceptible and resistant individuals of *P. nemorum* show a difference in host plant preference.

-Individuals of *P. nemorum* resistant to the defences of *B. vulgaris* prefer *B. vulgaris* as a host plant.

-*P. nemorum* individuals susceptible for the defences of *B. vulgaris* prefer *R. sativus* as a host plant over *B. vulgaris*.

-Host plant preference influences the population structure of *P. nemorum*, due to a limited spread between different host plants.

This experiment was designed to investigate whether the difference in R-gene frequencies between populations living on *B. vulgaris* and on other host plants, could be explained by the limited migration of flea beetles to different host plants caused by olfactory cues and host plant preference. If the resistant and the susceptible flea beetles showed no difference in preference it is unlikely that this is limiting migration between host plants and is the cause of different R-gene frequencies.
It was important to do a study about the preference, since it could be a major mechanism influencing the spread of the R-genes. The occurrence of these R-genes which allow the insect to feed on an extra host plant but which not all of the *P. nemorum* have, might be an indication that they are obtaining a new host plant. If this is the case it should be studied thoroughly since the next time a species develops a resistance, it might start eating on a crop plant and it might become a pest, or it could already be a pest species which becomes resistant to pesticides. The consequences of such a resistance could be very large in a financial way, but it could also result in a shortage of food.
If more research is done, it can result in an indication about a method to prevent or delay resistance forming and/or spread of the resistance through populations.

Material and methods

For this experiment a special olfactometer was used (figure 1) (Alvarez et. al., in press). It existed of a round cylinder with a diameter of 8.8 cm and 6 cm high, with a removable top. A plastic screen divided the cylinder in two equal parts. The bottom of the cylinder was made of a net, which enabled plant volatiles to go through to the lower part of the olfactometer. Below the net was a petridish (8.8 cm diameter and 1.5 cm high), with its open side pointing up. The lower compartment was used for the flea beetle. After the flea beetle was put in, parafilm was used to seal the two compartments together without leaving room for the flea beetle to escape. In the upper compartment the leaves of the plants were put. To ensure the plant would not be damaged by this set-up, the upper cylinder has two small openings on the top (one on each side) which allowed the stems of the leaves to go through without putting any pressure on them.

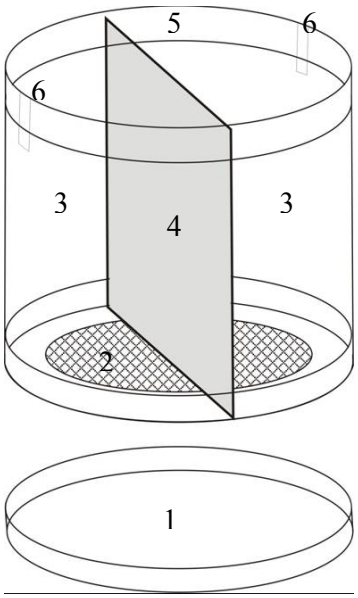

Figure 1, the olfactometer. The petridish (1) contains the flea beetle in the experiment, a net (2) separates it from the two compartments above (3). The two compartments, separated by a screen (4) and with removable lid (5), contain the leaves in the experiment with the stems of the leaves through small openings (6).

Two plant species were used in the experiment, *B. vulgaris* and *R. sativus*. The plants from *B. vulgaris* were about eight weeks old before they were used. The *R. sativus* plants were five to eight weeks old. In the experiment healthy leaves were used with approximately the same size. Leaves which looked damaged were excluded from the experiment. When possible new leaves were used for each different flea beetle. This was impossible for *B. vulgaris* because there were not enough plants with the right size of leaves. In the beginning of the experiment (testing *B. vulgaris* resistant flea beetles for preference for either *B. vulgaris* or *R. sativus*) this was also the case with *R. sativus* since only the largest leaves were big enough.

Two different populations of flea beetles were used in this experiment, a population of flea beetles which were resistant to the defences of *B. vulgaris* and a population of flea beetles which were susceptible for these defences. The populations had been reared in the lab for one or more generations before use in the experiment. The resistant flea beetles originally came from Kværkeby and the first and second reared generations were used. The susceptible flea beetles had been reared in the lab for many years. For rearing *R. sativus* was used with both populations, because this enables the flea beetles to reproduce better. To ensure the resistant flea beetles were still resistant in the experiment the larvae were fed with *B. vulgaris*; if any of the flea beetles would not have the genes for resistance it was unable to survive and thus would not be used in the experiment. The larvae of the susceptible flea beetles were fed with *R. sativus*. When the resistant larvae had turned into pupae, the leaves were removed and when adults emerged they were not fed before the experiment. In this way the adult flea beetles did not have any experience with one of the plants, avoiding any influence in the choice of the flea beetles. The emerged flea beetles were taken from the container every day, this way newly emerged flea beetles were used for each experiment. For the susceptible flea beetles this was not the case, there were still leaves in the container, but the *R. sativus* leaves were entirely dried and dead, and the big container with the leaves made it impossible to remove all of the flea beetles which had emerged. These flea

beetles might have been older when used in the experiment. Both the rearing and the experiment were conducted in a climate chamber with a temperature of about 23º Celsius.
With this set-up the following combinations were tested:
R. sativus versus an empty half with resistant flea beetles
B. vulgaris versus an empty half with resistant flea beetles
A green paper leaf versus an empty half with resistant flea beetles
B. vulgaris versus *R. sativus* with resistant flea beetles
B. vulgaris versus *R. sativus* with susceptible flea beetles
It was randomly chosen on which side the leaf was in the first three and which leaf was on which side in the last two tests.
The first three combinations were controls. The first and second combination showed whether flea beetles could detect a plant when they were in this set-up. The third made sure that the flea beetles did not use their eyes in the experiment but actually their olfactory senses. Otherwise the experimental set-up might not suffice to measure preference, since the flea beetles might just want to hide in some shade or are attracted by something green.
For every combination 40 flea beetles were used of which 20 were female and 20 were male.
A single flea beetle was put in the middle of the bottom part of the olfactometer, the leaves were put in the upper part. Both the beetles and the plants were put there one hour before the actual experiment started, this way the flea beetles had time to adjust to their surroundings and to calm down after putting them in there. After this hour had past, the flea beetles were monitored for one hour. The location of the flea beetles was written down at the start, after which it was recorded whether the flea beetles were sitting still, were walking around and when they switched from one side of the olfactometer to the other side. Except for the combination with *B. vulgaris* and *R. sativus* with resistant flea beetles also the jumping behaviour was recorded.
For the analysis the percentage of time on each side was used. This was converted into arcsin√percentage, before use. Besides using all data, also statistical tests were done without using the flea beetles which did not move. This was done because non-moving flea beetles might not have followed the same distribution as the other flea beetles and it could be the case that they just did not choose between the two sides.

Results

R. sativus *vs nothing*

The flea beetles tested for *R. sativus* against nothing tended to go to the *R. sativus* side a little more than to the other side (Table 2, Appendix 1). The tendency of the flea beetles to go to the radish (*R. sativus*) side was not significant (one sample t-test, t=0.601, N=40, p=0.552). Figure 2 shows the tendency of the flea beetles to go to the radish side. Most flea beetles were zero to five percent of the hour at the radish side. Then there is a big gap until 20 till 25 %. Twenty-four flea beetles out of 40 were on the radish side most of the time. Eight flea beetles stayed on the same side for the entire hour, of these eight only two were at the radish side. When removing the non-moving flea beetles, the remaining flea beetles show a significant preference for the *R. sativus* side (one sample t-test, t=2.861, N=32, p=0.007).

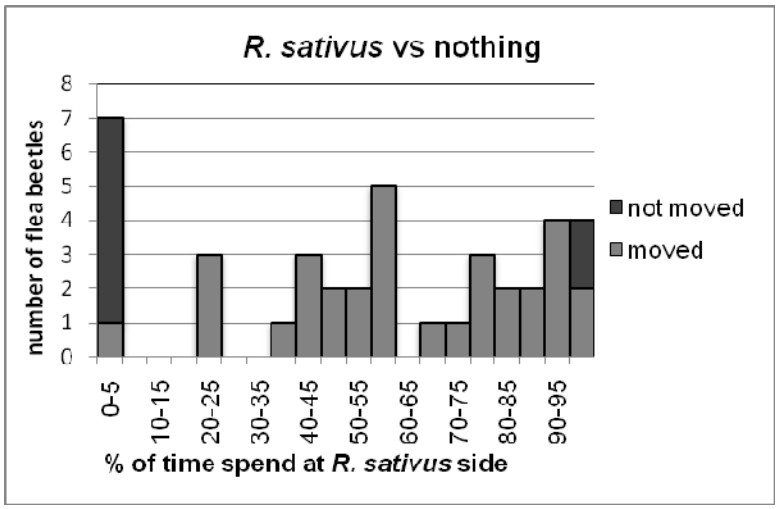

Figure 2, the percentage of time flea beetles spend at the *R. sativus* half of the olfactometer and not on the empty half.

B. vulgaris vs. nothing

The flea beetles tested for *B. vulgaris* versus nothing were significantly more on the *B. vulgaris* side (one sample t-test, t=3.846, N=40, p<0.001; Table 1, Appendix 1). Figure 3 shows the number of flea beetles that stayed at the *B. vulgaris* side for a percentage of time. One flea beetle did not go to the *B. vulgaris* side at all, while three flea beetles stayed on this side the entire time (one of them did walk some of the time). Most flea beetles stayed on the *B. vulgaris* side between 95 and 100 % of the time. Only six flea beetles out of 40 were seen more on the opposite side of *B. vulgaris*. High numbers of flea beetles were at the *B. vulgaris* side between 50 and 60 % of the hour. When the non-moving flea beetles were removed from the analysis, there was still a significant difference in distribution over the two halves of the olfactometer (one sample t-test, t=4.303, N=37, p<0.001).

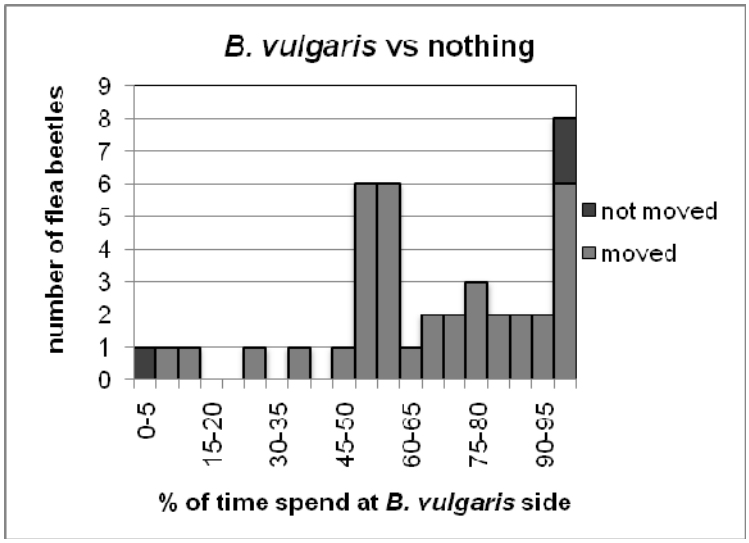

Figure 3, the percentage of time flea beetles spend at the *B. vulgaris* half of the olfactometer and not on the empty half

Paper leaf vs. nothing

The flea beetles tested for a paper leaf versus nothing, were not significantly more on either side, although they showed a tendency to go to the empty side (Figure 4; Table 3; Appendix 1; one sample t-test, t=-0.469, N=40, p=0.642). Also when the non-moving flea beetles, two on the paper side, were removed from the population they showed no preference (one sample t-test, t=-1.367, N=38 p=0.180). Seventeen of the flea beetles were more often on the side of the paper leaf, while the remaining twenty-three were more on the opposite side.

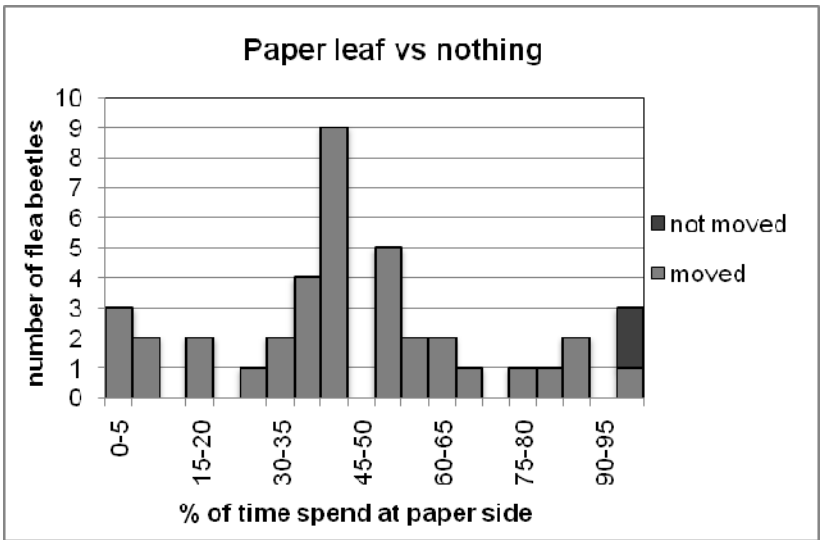

Figure 4, the percentage of time flea beetles spend at the paper leaf half of the olfactometer and not on the empty half.

Susceptible B. vulgaris *vs* R. sativus

The susceptible flea beetles, tested only for *B. vulgaris* versus *R. sativus* were not significantly more on either side (one sample t-test, t=1.071, N=40, p=0.291; Table 5; Appendix 1). On average they were more on the *B. vulgaris* side (Figure 5).

Two of the 40 flea beetles did not move, one was sitting on the *B. vulgaris* side, while the other was sitting on the *R. sativus* side. When removing these two flea beetles, they were still not significantly more on either side of the olfactometer (one sample t-test, t=1.136, N=38, p=0.263). Most of the flea beetles can be found in the group which stayed at the *B. vulgaris* side for 95-100% of the hour.

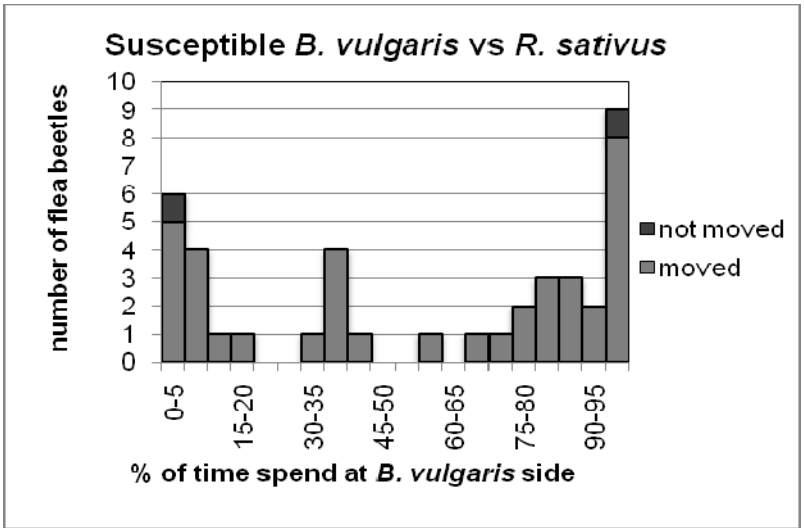

Figure 5, the percentage of time susceptible flea beetles spend at the *B. vulgaris* half of the olfactometer and not on the *R. sativus* side.

Resistant B. vulgaris *vs* R. sativus

The resistant flea beetles tested for *B. vulgaris* versus *R. sativus* were not significant more often at either side (one sample t-test, t=0.946, N=40, p=0.350; Figure 6; Table 4; Appendix

1). They do show a slight tendency to go to the *B. vulgaris* side. When the individuals which did not move during the entire hour were removed, the tendency to go to the *B. vulgaris* side and stay there became smaller (one sample t-test, t=1.280, N=32, p=0.210). Of the eight flea beetles which did not move, four were sitting on the *B. vulgaris* side during the test hour, the other four were on the opposite side the entire hour. The highest number of flea beetles can be found in the group being 95-100 % of the time on the *B. vulgaris* side, a second highest number of flea beetles was sitting on the *R. sativus* time for 95-100% of the time. When excluding the flea beetles which did not move, these numbers were down to respectively five and two beetles.

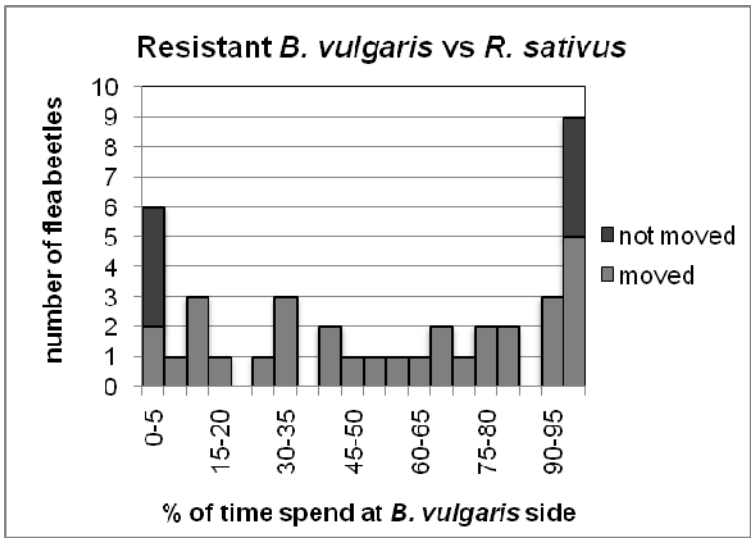

Figure 6, the percentage of time resistant flea beetles spend at the *B. vulgaris* half of the olfactometer and not on the *R. sativus* side.

When comparing the two tests with *R. sativus* versus nothing and *B. vulgaris* versus nothing, the difference was also not significant (independent sample t-test, t=-1.838, N=80, p=0.070), although the flea beetles tended to be a bigger part of the time on the side of *B. vulgaris* then on the side of *R. sativus*. When the non-moving flea beetles were removed, this is still the case (independent sample t-test, t=-0.780, N=69, p=0.438).

Susceptible vs. resistant

There appeared to be no difference in the distribution of the susceptible and the resistant flea beetles, when they were tested in the olfactometer with *B. vulgaris* and *R. sativus* (independent sample t-test, t=-0.092, N=80, p=0.927). With removal of the non-moving flea beetles this is also the case (independent sample t-test, t= 0.035, N=70, p=0.972).

Jumps

The number of jumps for each flea beetle had not been recorded for the test combination of *B. vulgaris* versus *R. sativus* with resistant flea beetles. Therefore this test combination was not used for these

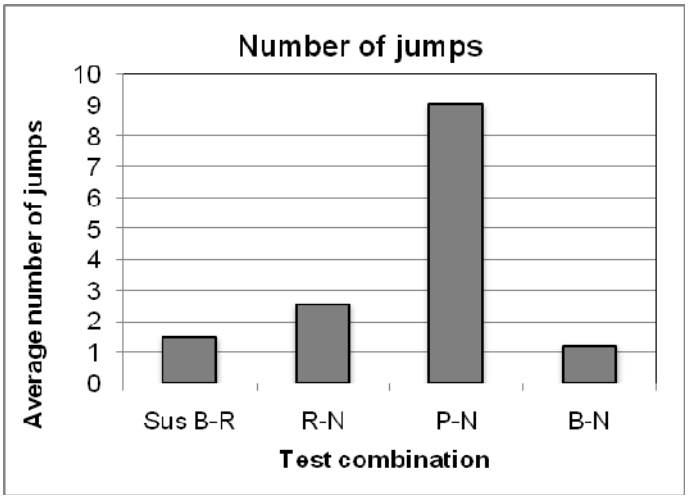

Figure 7, the average number of jumps in the different test combinations. In the first column the test was done with susceptible flea beetles, the others all with resistant flea beetles.

tests. The number of times the flea beetles jumped was significantly different between the groups (Kruskall-Wallis, χ^2=46.012, N=200, p<0.001). The average time a flea beetle jumped which was tested for a paper leaf versus an empty half was 9.05 times, while the other flea beetles jumped on average less than twice during the hour. The susceptibles tested for *B. vulgaris* versus *R. sativus* jumped 1.5 times, the flea beetles tested for *R. sativus* versus nothing 2.55 times and the flea beetles tested for *B. vulgaris* versus nothing 1.2 times (Figure 7). When testing the separate combinations, the paper leaf versus nothing was significantly different from all other combinations (Mann Whitney U tests, z=-3.792, N=40, p=<0.001; z=-2.786, N=40, p=0.005; z=-4.126, N=40, p<0.001). The others showed no significant difference with each other (Mann Whitney U test, z=-0.819, N=40, p=0.413; z=-0.510, N=40, p=0.610; z=-1.322, N=40, p=0.186).

Sex

When combining all the different combinations tested, the different sexes made a significantly different number of switches from one side of the olfactometer to the other (Mann Whitney U test, z=-2.134, N=200, p=0.033). The males were the ones switching most often from side. When testing the separately tested combinations, only the set-up with *R. sativus* vs nothing shows a significantly higher number of switches for the males (Mann Whitney U, z=-2.391, N=40, p=0.017). In all other combinations, though not significant the males did numerically switch more.

Conclusion and discussion

Olfactometer function

The flea beetles in both the test with *R. sativus* vs nothing and the test with *B. vulgaris* vs nothing showed a significant preference for the side with the leaf. This indicated that the flea beetles could detect the leaves from where they were. The data of the test with green paper against nothing was not normally distributed, therefore we should be careful with concluding things from the statistical tests. Because the tendency of the flea beetles was to go to the empty side, it is safe to say that the flea beetles showed no preference for the side with the green paper leaf. It is therefore very unlikely that the flea beetles chose a side based on the visual cues from the leaf in our experiment, the shape and colour of a leaf or the shade it produces did not seem to attract the flea beetles.

In most of the olfactometer experiments, for example in a Y-tube, there are two groups of test subjects, those that made a choice and those that did not make a choice. The ones that did make a choice moved to one of the arms of the Y-tube, those that did not make a choice would not go to one of the arms during a for that species selected period of time (Castrejon et. al., 2006; Pallini et. al., 1997). The ones that did not make a choice are not included in the statistical analysis. In this experiment no distinction was made between individuals that chose and individuals that did not chose. Individuals that did not make a choice are included in the data analysis. This could influence its outcome drastically, especially if large numbers of flea beetles did not choose. There were two possible actions for non-choosing flea beetles, either they sat still or they kept walking around through the olfactometer compartment. If the flea beetles kept moving around, they would have spend approximately the same amount of time on both sides. Therefore the influence on the statistics would not be too big if not too many flea beetles showed this behaviour, since the averaged time spent on both sides will only be a little closer to the 50%. Statistical tests have also been done with the non-moving flea beetles removed. If non-choosing flea beetles sat still, this is the data that should be used. The analysis with the non-movers removed seemed to make a small difference, which was especially important in the test with *R. sativus* versus nothing. In this test the big gap between 0-5 % and 20-25% of time the flea beetles spend at the *R. sativus* side in combination with the fact that most flea beetles in this first group were not moving might indicate that the non-moving flea beetles should indeed be removed. The non-moving flea beetles seemed to have an entirely different distribution than the moving flea beetles, therefore it is possible that the flea beetles should be divided in two groups, movers and non-movers, in which the movers were actively searching for food.

One reason for the flea beetles not to choose either side is that some flea beetles might not have been as hungry as others. This can be explained by the fact that not all flea beetles seemed to be able to emerge in the same time span. Some flea beetles were seen for a longer period of time in the jar under the vermiculite, while only pupae should stay there, adults should start digging up. Some of the adults did not manage to emerge at all. Another possible reason for non-movers is that the hour before the actual test began was too long. It is possible that the flea beetles already discovered that they could not reach the leaves of the plants they detected and stopped trying to get there and just decided to sit somewhere. Most non-moving flea beetles sat on the edge of either side of the olfactometer, this can be explained by the edge being a better hiding place than elsewhere in the olfactometer. They might not have wanted shade and something over their head, but something at one of their sides. Moving flea beetles did not always sit on the edge of the olfactometer compartment when they were not moving. In a number of cases the flea beetles sat directly under a leaf. That supports the idea that the non-moving flea beetles were not looking for something to eat at the moment.

Although there are a few questions to the method, the fact that highly significant results were obtained in the test with *B. vulgaris* versus nothing, while the paper leaf versus nothing gave no significant preference, supports the method of testing is a good method to test whether the flea beetles are attracted by the volatiles the plants produce and when using more than one plant whether they prefer one of the plants over the other.

Host-plant preference

One of the research questions was: Does *P. nemorum* prefer some host plants over others? With the results from this experiment it is impossible to answer this question. In the experiments with *B. vulgaris* and *R. sativus* all flea beetles seemed to have a tendency to go towards *B. vulgaris* and not towards *R. sativus*. This tendency was however not a significant preference. The flea beetles also showed a more significant preference for *B. vulgaris* over nothing than for *R. sativus* over nothing. The difference between these two tests was also not significant. The tendency to go to the *B. vulgaris* side may indicate that there was a preference, but only a small one. It is possible however that this tendency is only the result of too few samples. With a small number of samples even one additional sample can make a difference.

Another thing one must think about is that *B. vulgaris* and *R. sativus* are not the only host plants for *P. nemorum*. It is possible that for example *S. arvensis*, the most common host plant of *P. nemorum,* is preferred over both plant species used in the experiment.

Also remarkable is that tests conducted in the dark gave more significant results than those in light conditions (D. C. Hernandez, personal communication). This leads to the question whether it is possible that the flea beetles are just as active or more active at night. It is possible that the flea beetles tend to migrate in the dark. This could have advantages, because for example most predators are asleep then. The probability of night time migration is reduced by the tendency of the flea beetles to be less active when the temperature is lower (personal observations) Another possibility is that the flea beetles tend to hide and sleep at the host plant and therefore tests in dark can give more significant results.

Susceptible-resistant

Both susceptible and resistant flea beetles tended to go to the side with *B. vulgaris* in the experiment. It seems somewhat strange that the susceptible flea beetles tended to go to the side of *B. vulgaris*, while eating this plant would kill them. An explanation for this could be that the leaf volatiles of both *B. vulgaris* and *R. sativus* did indeed attract the flea beetles at first, but before eating a leaf the flea beetles might normally investigate the leaf first. Henderson, Hallett and Soroka (2004) have shown that other insects, like the related *Phyllotreta cruciferae* follow a series of prefeeding behaviour before starting to feed. This prefeeding behaviour consists of both tarsal and antennal touching of the leaves. This way the insects can gather more information about the leaf by the chemicals in its wax layer. The further investigation of the leaves was impossible in the set-up used in this experiment. It is possible that the susceptible flea beetles would start looking for another host plant after they have had time to investigate the plant. The research questions: do susceptible *P. nemorum* have a preference for *R. sativus* over *B. vulgaris* and do resistant *P. nemorum* have a preference for *B. vulgaris* as a host plant, are therefore not as easy to answer as it seems. The flea beetles did not show a preference based on olfactory cues only.

Since both susceptible and resistant flea beetles showed a slight tendency to go to the *B. vulgaris* side and the difference between the two groups was negligible, the research question; is there a difference in preference between individuals which are susceptible and resistant to the defences of *B. vulgaris,* seems to be easy to answer. As stated before however only the preference based on volatiles has been tested, preference based on other chemicals is also

possible. We should not forget either that there are also other host plants. Flea beetles from the different groups, susceptible and resistant may show differences in preference for these other plants.

Sex
The males switched more often from one side to the other side than the females did. This was the case in all combinations, but only significant if all were taken together, except for the test with *R. sativus* against an empty side. The reason for this can be simply a matter of sample size, since only twenty males and twenty females were used for every test combination. The males seemed to be more active in this experiment than the females were. It is possible that males are generally more active or that were not only looking for food, but also for a mate, which made them more eager to look around.

Jumps
The test using paper leaves on the one half and nothing on the other half of the olfactometer showed a remarkably high number of jumps made by the flea beetles. An explanation for these jumps could be that the flea beetles did not detect any host plants in their surrounding and therefore wanted to migrate as far and fast as possible, to find a plant they can feed on. A major way for *P. nemorum* to migrate is by jumping (De Jong, De Vos and Nielsen, 2001).
Another possibility is that the paper also contained some substances which the flea beetles could detect and which made them more active. If it repelled them it would explain the jumping behaviour since this is their main method of migration. In this case it would also be likely that the flea beetles were found more in the side without the paper leaf. The flea beetles were indeed more of their time in the empty side of the olfactometer, but this was not significant. If the flea beetles became more active because of a component of the paper, this could explain the high number of jumps, but then the number of switches from one side to the other would also expected to be higher and this is not the case. Therefore it is unlikely that this plays a role.

Population structure
The final research question was: Does host plant preference influence the population structure of *P. nemorum*?
This question is not easily answered. In this experiment no evidence for preference was found, though some tendency was. The tendency may be a small preference for *B. vulgaris*, but this is not proven. It might be important to also perform the test with the most important host plant, *S. arvensis*. This is the host plant where *P. nemorum* is found on most often, therefore it is likely that this is the host plant flea beetles prefer over all others. The fact that no significant difference in preference between resistant and susceptible flea beetles is found makes it less likely that the population structure of the flea beetles is determined mainly by host preference. But since preference can also occur in a later stadium of host plant selection it is possible that there is a preference but it is undetected by this method of testing. Whether the preference influences the population structure and thereby the distribution of the R-genes remains unknown.

It is necessary to conduct more research to be able to answer the research questions. This research could exist of olfactometer tests in the dark, olfactometer tests with more plant species and more flea beetles, more basic observations about flea beetles migration and preference tests in which the flea beetles can touch the leaves.

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Appendix 1 results

Table 1, Preference of resistant *P. nemorum* for *B. vulgaris* versus nothing. The column most on the left indicates the number of the flea beetle, the second column states the percentage of the hour test time that the flea beetle was on the side of *B. vulgaris.* The third column indicates the number of times the flea beetle switched from one side to the other side of the olfactometer. The fourth column states the sex of the flea beetle and the fifth column whether the flea beetle moved, or sat still the entire time. The last column states the number of jumps the flea beetle made during the hour.

flea beetle nr.	% at *B. vulgaris*	switches	sex	movement	jumps
1	52,5	11	male	moved	0
2	52,22222	75	male	moved	1
3	55,83333	46	male	moved	0
4	53,33333	78	male	moved	0
5	53,05556	79	female	moved	0
6	38,88889	20	male	moved	0
7	45,27778	50	male	moved	0
8	81,66667	2	female	moved	0
9	70,97222	12	female	moved	3
10	93,33333	9	female	moved	2
11	100	0	female	moved	0
12	15	17	female	moved	1
13	8,333333	6	female	moved	0
14	82,08333	35	female	moved	4
15	56,38889	51	female	moved	0
16	51,80556	54	female	moved	0
17	96,11111	9	male	moved	0
18	29,44444	65	male	moved	2
19	77,5	35	male	moved	0
20	62,77778	20	male	moved	4
21	76,66667	14	male	moved	7
22	55,55556	47	male	moved	0
23	69,72222	61	female	moved	8
24	58,75	84	female	moved	4
25	89,16667	9	female	moved	1
26	98,33333	2	female	moved	0
27	54,44444	54	female	moved	3
28	98,88889	2	female	moved	1
29	0	0	female	none	0
30	77,77778	30	female	moved	0
31	100	0	female	none	0
32	58,88889	54	female	moved	0
33	70	27	male	moved	0
34	87,5	8	male	moved	3
35	99,16667	2	male	moved	0
36	69,16667	41	male	moved	0
37	90	22	male	moved	4
38	100	0	male	none	0
39	98,33333	4	male	moved	0
40	56,11111	77	male	moved	0

Table 2, Preference of resistant *P. nemorum* for *R. sativus* versus nothing. The column most on the left indicates the number of the flea beetle, the second column states the percentage of the hour test time that the flea beetle was on the side of *R. sativus.* The third column indicates the number of times the flea beetle switched from one side to the other side of the olfactometer. The fourth column states the sex of the flea beetle and the fifth column whether the flea beetle moved, or sat still the entire time. The last column states the number of jumps the flea beetle made during the hour.

flea beetle nr.	% at *R. sativus*	switches	sex	movement	jumps
1	45,83333	34	male	moved	0
2	71,66667	16	male	moved	0
3	78,33333	24	female	moved	5
4	59,72222	74	male	moved	2
5	87,5	12	male	moved	3
6	0	0	female	none	0
7	80,83333	30	female	moved	0
8	100	0	female	none	0
9	3,333333	6	female	moved	0
10	22,61111	46	female	moved	1
11	43,61111	13	female	moved	3
12	24,44444	22	female	moved	0
13	75,83333	11	female	moved	2
14	54,44444	54	male	moved	3
15	58,61111	62	male	moved	5
16	92,77778	6	male	moved	0
17	41,5	81	male	moved	17
18	56,38889	97	male	moved	1
19	68,33333	28	male	moved	0
20	58,05556	45	male	moved	0
21	97,5	6	male	moved	0
22	0	0	female	none	0
23	100	0	female	none	0
24	79,44444	12	female	moved	7
25	94,58333	7	female	moved	8
26	99,16667	2	female	moved	0
27	53,05556	30	female	moved	6
28	0	0	female	none	0
29	88,88889	9	female	moved	1
30	59,16667	56	male	moved	3
31	0	0	male	none	0
32	93,33333	7	male	moved	0
33	46,94444	26	male	moved	0
34	39,30556	52	male	moved	11
35	0	0	male	none	0
36	91,66667	20	male	moved	0
37	83,33333	34	male	moved	9
38	21,38889	27	female	moved	5
39	44,16667	70	female	moved	10
40	0	0	female	none	0

Table 3, Preference of resistant *P. nemorum* for a paper leaf versus nothing. The column most on the left indicates the number of the flea beetle, the second column states the percentage of the hour test time that the flea beetle was on the side of the paper leaf. The third column indicates the number of times the flea beetle switched from one side to the other side of the olfactometer. The fourth column states the sex of the flea beetle and the fifth column whether the flea beetle moved, or sat still the entire time. The last column states the number of jumps the flea beetle made during the hour.

flea beetle nr.	% at paper leaf	switches	sex	movement	jumps
1	41,25	3	female	moved	7
2	100	0	female	none	0
3	37,05556	51	female	moved	2
4	82,77778	19	female	moved	15
5	52,5	50	female	moved	5
6	55,83333	20	female	moved	1
7	50,27778	79	female	moved	13
8	5,972222	12	female	moved	0
9	64,16667	35	male	moved	1
10	86,94444	12	male	moved	1
11	16,94444	26	male	moved	0
12	0,555556	2	male	moved	0
13	100	0	male	moved	0
14	62,77778	21	male	moved	4
15	40,83333	37	male	moved	7
16	0	0	male	moved	1
17	39,94841	121	male	moved	77
18	3,333333	5	male	moved	0
19	27,91667	47	male	moved	17
20	43,75	53	male	moved	7
21	42,63889	56	female	moved	12
22	37,22222	62	female	moved	6
23	58,75	54	female	moved	30
24	100	0	female	none	0
25	53,75	49	male	moved	14
26	65,83333	51	male	moved	10
27	53,33333	84	male	moved	9
28	33,9246	76	male	moved	90
29	19,16667	24	female	moved	1
30	40,83333	47	female	moved	2
31	42,22222	3	female	moved	4
32	43,05556	54	female	moved	2
33	54,72222	57	female	moved	0
34	41,38889	26	female	moved	10
35	43,61111	25	female	moved	3
36	5,555556	11	female	moved	1
37	34,16667	71	male	moved	1
38	86,66667	2	male	moved	3
39	77,63889	21	male	moved	1
40	38,05556	19	male	moved	5

Table 4, Preference of resistant *P. nemorum* for *B. vulgaris* versus *R. sativus*. The column most on the left indicates the number of the flea beetle, the second column states the percentage of the hour test time that the flea beetle was on the side of *B. vulgaris.* The middle column indicates the number of times the flea beetle switched from one side to the other side of the olfactometer. The fourth column states the sex of the flea beetle and the last one indicates whether the flea beetle moved, or sat still the entire time.

flea beetle nr.	% at *B. vulgaris*	switches	sex	movement
1	100	0	female	moved
2	93,33333	6	female	moved
3	0	0	female	none
4	43,33333	30	male	moved
5	93,33333	6	female	moved
6	13,88889	9	female	moved
7	81,66667	11	female	moved
8	29,16667	11	female	moved
9	8,333333	1	female	moved
10	68,33333	49	female	moved
11	100	0	female	none
12	40,27778	67	male	moved
13	0	0	male	none
14	81,66667	2	male	moved
15	100	0	female	moved
16	91,66667	9	female	moved
17	100	0	female	none
18	100	0	female	moved
19	69,16667	27	female	moved
20	35	20	female	moved
21	78,33333	1	female	moved
22	3,333333	2	female	moved
23	98,33333	2	male	moved
24	52,77778	96	female	moved
25	0	0	female	none
26	61,66667	10	male	moved
27	35	7	male	moved
28	57,22222	89	male	moved
29	78,33333	7	male	moved
30	100	0	male	none
31	15	21	male	moved
32	0	0	male	none
33	100	0	male	moved
34	0	0	male	moved
35	13,33333	13	male	moved
36	16,38889	20	male	moved
37	100	0	male	none
38	34,44444	24	male	moved
39	75	17	male	moved
40	46,94444	76	male	moved

Table 5, Preference of susceptible *P. nemorum* for *B. vulgaris* versus *R. sativus*. The column most on the left indicates the number of the flea beetle, the second column states the percentage of the hour test time that the flea beetle was on the side of *B. vulgaris.* The third column indicates the number of times the flea beetle switched from one side to the other side of the olfactometer. The fourth column states the sex of the flea beetle and the fifth column whether the flea beetle moved, or sat still the entire time. The last column states the number of jumps the flea beetle made during the hour.

flea beetle nr.	% at *B. vulgaris*	switches	sex	movement	jumps
1	100	0	male	moved	0
2	99,16667	2	female	moved	0
3	8,333333	9	male	moved	4
4	100	0	male	moved	0
5	100	0	female	moved	0
6	37,5	29	female	moved	3
7	100	0	male	moved	0
8	0	0	male	moved	0
9	96,66667	2	male	moved	0
10	100	0	female	moved	0
11	93,33333	1	female	moved	0
12	6,666667	4	female	moved	2
13	86,66667	4	male	moved	3
14	80	3	female	moved	0
15	0,833333	2	male	moved	1
16	100	0	female	moved	0
17	0	0	female	none	0
18	17,5	4	female	moved	1
19	36,66667	2	male	moved	0
20	10	2	female	moved	1
21	94,44444	9	male	moved	0
22	85,55556	23	male	moved	18
23	69,16667	5	female	moved	1
24	88,33333	5	female	moved	1
25	38,33333	1	male	moved	0
26	31,66667	3	female	moved	0
27	85	3	female	moved	3
28	81,11111	13	male	moved	0
29	7,5	4	male	moved	3
30	3,333333	4	male	moved	1
31	43,33333	1	male	moved	1
32	36,66667	19	female	moved	4
33	5	3	female	moved	0
34	0	0	female	moved	1
35	71,11111	16	male	moved	0
36	100	0	female	none	0
37	60	5	male	moved	2
38	11,66667	6	female	moved	1
39	78,88889	18	male	moved	9
40	85	8	male	moved	0

Appendix 2 Barbarea toxicity

During and before the experiment *B. vulgaris* plants were infested with aphids. Before using these plants it was necessary to conduct a test to see if the plants were still toxic for susceptible flea beetles and thus usable.

Objective:
Testing whether leaves of *B. vulgaris* infested with aphids are still toxic for susceptible *P. nemorum.*

Material and methods:
A white filter paper was put into a petridish with a lid (8.8 cm diameter) and moistened a little. A circle of a *B. vulgaris* leaf was put in the middle after which a susceptible unfed larva of *P. nemorum* was put on top of it. This was done with fifteen larvae, after which the number of living and dead *P. nemorum* was written down the next day, as well as whether the flea beetle was inside the leaf.

Results:
The results are shown in table 6. Of the fifteen larvae two were missing the next day. None of the larvae had started to mine the leaf disc. Eight of the larvae were already dead when this was checked and five were still alive.

Table 6, Survival of flea beetle larvae on *B. vulgaris*. The first column gives the state of the larvae, the second column the number of larvae in this state.

	Number of larvae
alive	5
dead	8
in leaf	0
lost	2

Conclusion & Discussion:
Since none of the larvae started mining in the leaf discs it is probable that the leaves are still toxic to *P. nemorum* after aphids started feeding on them. The fact that eight of the larvae died supports this.
The two lost larvae could have walked out of the set-up between the petridish and its lid. The larvae are quite small and need only little room to slide through.

Recommendation:
The leaves of these *B. vulgaris* plants are suitable to use in further experiments, they are still toxic to susceptible flea beetles.

Appendix 3 Marking

At first the idea was to investigate migration in the field with a mark-recapture experiment in Denmark. This way we could see how far a flea beetle migrates in a specific amount of time. For this mark-recapture experiment we would have to find a suitable way of marking.

Objective:
Finding a paint which is easy applicable and stays on the flea beetle for approximately two weeks.

Material and methods:
In this experiment flea beetles from the stock in both Wageningen and Denmark have been used. In Wageningen we marked five flea beetles by using Bruna correctie vloeistof (type-ex) in a small bottle with a brush attached to the lid. We also used pink Hema fluorverf on five beetles. A hair from a dishwashing brush was used to put the paint and the type-ex on the elytra of the flea beetles. Also five flea beetles were marked using a black Nashua cd/dvd writer pen and five with a blue edding 3000 permanent marker (made in Germany). Both were used on the yellow part of the flea beetles elytra to make sure it was visible. The flea beetles were taken to a cold room of 6ºC, where they were marked. The low temperature calms the flea beetles down, so they do not jump and run as much as at a higher temperature. The flea beetles were put back in the container with *R. sativus* after marking. After four days we checked 101 flea beetles in the container to see if we could find markings. Another attempt to find a suitable paint was done using a red Hoogglans Brillant (Gamma extra dekkende lakverf) and light blue Hamerslaglak (Gamma) paint on five flea beetles each. These were also marked in the cold room and put in a separate container with *R. sativus*. After three days we checked them to see if they still carried their paint. A third attempt was done using uniPOSCA (bullet tip/fine line) fade and water resistant paint (pat. Mitsubishi pencil co ltd. and made in Japan). Five flea beetles were marked with the red acrylic paint on the elytra in the cold room. After marking the flea beetles were kept in a container with *R. sativus* for more than a week. In Denmark we marked five flea beetles with the same uniPOSCA paint, but now in the colours: bright yellow, light blue, green, pink and violet. We used again a cold room (5º C) to mark the flea beetles. The flea beetles were put in a small plastic container with a few leaves of *R. sativus*. We checked the flea beetles for paint after four, five, seven, eight and nine days. After this we did a test marking five flea beetles each with one of the six colours of uniPOSCA paint. This was done the same way as before. The flea beetles were put with two colours together in a small container containing *R. sativus*. The colour combinations were: green and pink, violet and bright yellow, light blue and red. The flea beetles were checked for marks after two, three and four days. Additional to this we also tried a different acrylic paint. The red paint was applied the same way as the uniPOSCA paint at five flea beetles. Also pink fluor powder was used on five flea beetles. The flea beetles were put in a plastic bag with this powder and remover right after putting them in. The flea beetles had already rolled through the powder which made it attach to their entire body. The flea beetles marked with the powder and the red acryl paint were checked on the day after marking and on the day after this. At the end a small test was done using four flea beetles, a small peace of sandpaper to roughen the elytra and yellow uniPOSCA paint. Also five flea beetles had one of the antennae partly removed. This was done using a nail clipper. The ten flea beetles were put in a small container with *R. sativus* to feed on. They were checked after one, four, and five days.

Results:

In the first part, with the type-ex, pink fluor paint, cd/dvd pen and the permanent marker, only one marked flea beetles was found after four days (Table 7). This flea beetle had been marked with the cd/dvd pen. The fluor paint was easiest to apply, while the cd/dvd pen was the most difficult. Except for de cd/dvd pen all methods resulted in flea beetles sticking to the marking device. Both with the cd/dvd pen and the permanent marker it was difficult not to crush the flea beetle while marking. The ink of the permanent marker did not want to stick to the flea beetle. Sometimes the flea beetle stuck to the marker and after carefully removing them from each other, the flea beetle did not show a colour on its elytra. The fluor paint needed the longest time to get dry.

Table 7, Marking of *P. nemorum* with type-ex, fluor paint, a cd/dvd pen and a permanent marker. The first column states the type of paint used to mark the flea beetles. The second column states the way the marking went. Sticky means in this table that the flea beetles tended to stick to the paint which was still on the dishwashing brush hair or the marker. Difficult means it was difficult to mark the flea beetle in a way that the mark was visible, while easy means it was not difficult. If it says needs to dry, it took a little while before the paint was dry, in this time the flea beetles spread the paint over things they touched. Crushes flea beetles indicates that it was quite difficult to mark the flea beetle without crushing it, since some pressure was needed to get the ink on the beetles. The third and last column states the number of flea beetles found with a specific marking in 101 recaptured flea beetles after four days.

Type of mark	method	markings found
type-ex	sticky, difficult	0
fluor paint	needs to dry, sticky, easy	0
cd/dvd pen	difficult, crushes flea beetles	1
permanent marker	crushes flea beetle, very difficult, sticky, doesn't attach to flea beetle	0

The ten flea beetles marked with the red and light blue Gamma paints were, although put in a separate container largely impossible to find after three days. One flea beetle was recovered but this one did not have any markings.

The test in which red uniPOSCA paint was used in Wageningen was performed next. Unfortunately no exact data was recorded. It seemed however that the paint would stay on the elytra for a sufficient amount of time (more than a week).

In the test using five different colours of uniPOSCA paint, the fourth day only three flea beetles carried their mark, the violet, bright yellow and light blue ones. The day after this only the violet and the blue flea beetle had their colour. The seventh day only one was marked, the mark was violet. The violet colour stayed on until the flea beetle died on the ninth day.

In the test with marking five flea beetles with each of the six uniPOSCA colours, there was some variation. The green paint lost its colour on four flea beetles before the second day, the last flea beetle had lost its colour on the fourth day (table 8). The pink marked flea beetles were all without mark on the third day, one of them still had its colour on the second day after marking. The violet paint was one of the paints lasting longest. After two and three days still two flea beetles were marked. After four days one still carried its mark. The bright yellow marked flea beetles are the others keeping the mark long. After two days three were still marked, after three days two and after four days still one. The light blue paint stayed on the flea beetles for a short period. At the second day only one flea beetle was marked, and at the third day all five had lost their colour. The red paint had the highest number of marked

individuals at day two, four flea beetles showed a red spot. At the third day however only two showed a mark and the fourth day all were without mark.

Table 8, the results of marking *P. nemorum* with uniPOSCA acrylic paint. The first column indicates the colour of the paint used. The second, third and fourth column give number of flea beetles with a mark on respectively the second, third and fourth day.

colour	day 2	Day 3	day 4
green	1	1	0
pink	1	0	0
violet	2	2	1
bright yellow	3	2	1
light blue	1	0	0
red	4	2	0

The next test included the red acrylic paint and the fluor powder. After one day the fluor powder was already invisible. The acrylic paint still had one marked flea beetle left. This marked flea beetle was unmarked the next day.

A final test was done with roughening the elytra and using bright yellow acrylic paint and with flea beetles with partly removed antennae. The paint stayed on for at least five days. The markings on one of the flea beetles was however hardly visible on the last day. The flea beetles with partly removed antennae were all still alive at the last day. Some of the leaves had also been eaten.

Conclusion & Discussion:
The methods of keeping the flea beetles in the first two experiments were unfit ways. In the first one it was impossible to distinguish between flea beetles which had been marked and flea beetles which had not been marked. The large number of flea beetles in the container also made it impossible to know if the marked flea beetles were captured to check their mark. In the second test the container the flea beetles were put in was still big enough to make it impossible to find all flea beetles. Since in both these test we could not be sure if the marks were gone on the flea beetles which were not found, they were not really good tests. In the other experiments smaller containers were used.
None of the used paints seemed to stay on the flea beetles long enough, although at first it seemed to be that way for the uniPOSCA paint. The only two methods of marking which lasted long enough were cutting of a part of the antennae and roughening the elytra and painting afterwards. Since the following experiment was meant to investigate migration of the flea beetles, we decided that cutting of a part of the antennae is unsuitable. The flea beetles might not be able to find new host plants after a part of their antennae is cut of. Also roughening the elytra before painting is unsuitable because this would take too much time for every flea beetle
For doing a mark-recapture test new methods for marking flea beetles have to be tested, since none of the methods used in these tests was qualified.

Appendix 4 Pilot release experiment

The original plan of the mark-recapture experiment was unfeasible, because the paint did not last long enough and because the weather was unsuitable for capturing many flea beetles at one day. Another plan for an experiment about migration in the field was made. In this experiment also marking and releasing was used, but now the paint only had to last for a couple of days. Before being able to perform the experiment the best way of releasing the flea beetles had to be investigated. The performed experiment is described in Appendix 5.

Objective:
This pilot experiment was done to see what way of releasing flea beetles would work best in a release and recapture experiment.

Material and methods:
Four plants of *Brassica nigra* were put in four different directions at approximately 0.5 meter from the point of release, as shown in figure 8.
Brassica nigra was used in this pilot because this plants is a suitable host plant for *P. nemorum* and it contains allylisothiocyanate (CH_2=$CHCH_2NCS$) this is a volatile which might attract *P. nemorum* (J. K. Nielsen, personal communications).

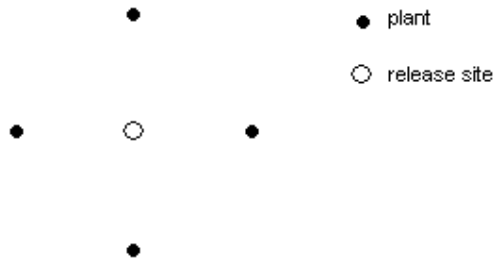

Figure 8, experimental set-up. This figure shows the set-up of the experiment. In the middle is the release site (open circle). On the four sides there stand *B. nigra* plants.

Different release methods were investigated, a method at which the flea beetles should not be stressed, a method of half-stress and a method with stress. The non-stressed method was putting the jar with the flea beetles at the point of release half an hour before releasing. The half-stressed method was putting the jar with the flea beetles at the point of release and opening it right after putting it there. The stressed method was opening the jar, holding it upside down and hitting it against the ground until all flea beetles were out.
These methods of release were tested with leaves of *B. nigra* in the pots with the flea beetles and without leaves in the pot, to see if it was better to release the flea beetles on a plant or not on a plant. All of these methods were done with flea beetles which were able to feed until the test, with flea beetles which had not been allowed to feed for one day and with flea beetles which had not been allowed to feed for three days.
In the pilot approximately ten flea beetles were released, after which the time was written down every time a flea beetle moved away from the point of release, this was done until five flea beetles had moved or 20 minutes had passed.

Results & Conclusions:
Table 9 to 11 give the results of this pilot. There is no data for the well fed flea beetles released with a leaf and with half stress, the flea beetles were not sitting on the leaf and therefore the data would be the same as when they did not have leaves. The following days we put some leaves in the pots before starting the test, this way they had some time to start sitting on the leaves. In the experiment with the non stressed flea beetles, this was already the case since we put them in the release circumstances half an hour before release. Flea beetles released with stress seemed to disperse quicker than flea beetles released without stress.

Between half-stress and non-stress there was not much difference. The difference between flea beetles released with leaves and without leaves was very small. It seemed that additional circumstances like the weather had more influence on the time the flea beetles waited before leaving the release point. Not feeding the flea beetles for a couple of days seemed to make the flea beetles more eager to go away, but this could also seem to be this way because of the weather.
Although it is until now assumed that flea beetles rarely fly, in this experiment quite some flea beetles flew. Both in non-stressed and in stressed circumstances flea beetles flew, also well fed and three days non-fed flea beetles flew. It is striking that there was one day in which no flea beetles flew, this can be caused by the weather. The surroundings can also influence the decision of flea beetles to fly, but since only little is known about the flight behaviour of flea beetles, more research should be done.

Recommendations: For the next experiment the best thing to use is half-stressed release without leaves or plants. Since other influences like the weather has more influence on the departing of the flea beetles than whether they have had food or not, it will depend on the experiment whether the flea beetles should be fed or not.

Table 9, the time between releasing the well fed flea beetles and its departing from this site. The first column indicates how many flea beetles were released. The second and third indicate whether the flea beetles were released with stress, half-stress or without stress and with or without leaves. The fourth till eight column indicate the times at which the first five flea beetles left. The last column gives remarks.

nr of flea beetles	Release circumstances		time of leaving flea beetle					remarks
11	non-stress	with leaf	7:24					1 flew away
11		without leaf	5:15	6:23	6:23	9:45	20:22	3 flew away
10	half-stress	with leaf	No data					
11		without leaf	2:30	3:20	8:30	8:40	13:00	
10	stress	with leaf	0:50	1:48	3:20	4:03	5:40	1 flew away
10		without leaf	0:11	0:16	0:16	0:46	0:48	2 flew away

Table 10, the time between releasing the flea beetles which have been without food for one day and its departing from the release site. The first column indicates how many flea beetles were released. The second and third indicate whether the flea beetles were released with stress, half-stress or without stress and with or without leaves. The fourth till eight column indicate the times at which the first five flea beetles left. The last column gives remarks.

nr of flea beetles	Release circumstances		time of leaving flea beetle					remarks
10	non-stress	with leaf	0:09	0:17	9:30			
9		without leaf	2:10	4:20				
9	half-stress	with leaf	13:15					
10		without leaf	10:00					
10	stress	with leaf	3:43	9:00	11:50			
10		without leaf	0:20	6:22	12:00			

Table 11, the time between releasing the flea beetles which have been without food for three days and its departing from the release site. The first column indicates how many flea beetles were released. The second and third indicate whether the flea beetles were released with stress, half-stress or without stress and with or without leaves. The fourth till eight column indicate the times at which the first five flea beetles left. The last column gives remarks.

nr of flea beetles	Release circumstances		time of leaving flea beetle					remarks
10	non-stress	with leaf	5:50					
10		without leaf	0:23	0:23	0:33	0:43	2:33	
10	half-stress	with leaf	0:53	2:35	4:04	4:49	12:30	
10		without leaf	0:13	0:30	0:40	0:56	1:25	
10	stress	with leaf	0:05	0:05	0:10	0:20	0:20	before 1 minute all gone but one
9		without leaf	0:27	0:39	1:30	3:07	3:07	3 flew away

Appendix 5 Alternative release-recapture experiment.

This experiment was performed as an alternative to the planned mark-recapture experiment. The planned experiment could not take place because the paint did not last long enough and the weather was not suitable to capture and mark hundreds of flea beetles at one day. Therefore this experiment was performed.

Objective: Determine how far *P. nemorum* spreads in a specific amount of time, whether plant species and weather conditions affect the migration speed.

Material and methods:
In Taastrup in Denmark an area was used with remnants of wheat (max 10 cm high) on it, this way normal conditions were mimicked. On this field three concentric circles were made by putting a stick in the middle, holding one end of a measure tape to it and putting a plant at the other end. Walking around the centre two plants were put down every meter (figure 9). The circles were made with radii of 10, 20 and 30 metres, therefore 400 plants were needed. The plants used in this experiment were *R. sativus* and *Brassica nigra,* they were grown in a greenhouse for three weeks before use. These two plant species were used because *B. nigra* produces a lot of glucosinolates, while *R. sativus* does not. A glucosinolate is a volatile which might make the plants easier to detect by *P. nemorum*. The plants stood in square cardboard cups of 5 cm high, 4.5 cm wide at the top and 2.5 cm wide at the bottom. This way the plants were elevated slightly over the surroundings.

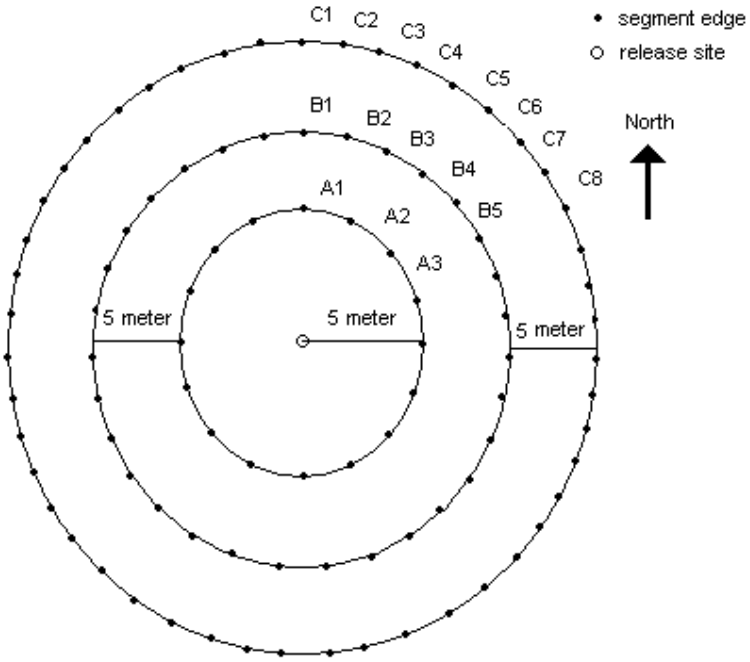

Figure 9, Schematic experimental set-up release-recapture experiment. The middle is the release site, surrounding this are three circles, the inner one (A-circle) with a diameter of 10 meter, the middle circle (B-circle) has a diameter of 20 meters and the outer one (C-circle) of 30 meter. Between two black dots is a segment of a circle. Within these segments four plants are standing with a distance of 50 cm from each other. The north side marks the beginning of the first segment in all circles, A1 in circle A, B1 in circle B and C1 in circle C. The circles are that way named with following numbers clockwise, A1 till A16, B1 till B31 and C1 till C47.

Every day 200 flea beetles were used. These flea beetles came from the stock in Denmark or were collected as larvae for an experiment about parasitoids. First the flea beetles were taken to a cool cell (5°C) to make them less active. In this cell they were marked with uniPOSCA paint on the elytra, different colours each day. Two jars with each 100 flea beetles were painted and taken to the field. The 200 flea beetles were then released by putting two jars each containing 100 (marked) flea beetles in the centre and taking of the lids.

The three circles were divided in smaller parts, each containing four plants and approximately two metres long. Every day two lists were printed with randomly chosen one of the parts of one of the circles for every ten minutes. To ensure that the smaller circle was monitored as often as the bigger circles, the smallest circle counted six times, the middle circle counted three times and the largest circle counted twice in the random choice.
For ten minutes one part was monitored, collecting the flea beetles in this part and writing down which colour they had after this time the flea beetles were released and the next part standing on the list was monitored. The monitoring started directly after releasing the flea beetles and was done by two persons, which made it possible to have two observations at every moment at two different locations on the three circles. First day the experiment was done with unmarked flea beetles and *R. sativus*. The second day the flea beetles from the stock were marked red, while the flea beetles collected from the field were marked pink. The third day the *R. sativus* was replaced by *B. nigra*, after which bright yellow marked flea beetles from the stock and blue marked flea beetles from the collection were released. The fourth day green marking was used for the stock flea beetles and violet marking for the other flea beetles. The weather conditions were monitored each day.
The day the *R. sativus* plants were changed for *B. nigra* plants, the plants were checked for flea beetles and the flea beetles checked for colour. The last day all plants were retrieved, and every plant was checked again for flea beetles. The flea beetles found were also checked for colour and this was written down.

Results:

The results are visible in table 12. Weather conditions consultable on request.
The first day the weather prohibited an entire watch of four hours. After watching two hours and 20 minutes the rain started to become thunder. The decision was made to stop the experiment for that day because no flea beetles had been found yet and the weather did not make it more likely that the flea beetles would migrate.
The last two days also a slightly shorter period was watched. The twenty extra minutes were used for replacing and retrieving plants. The second day only a few flea beetles were found, all were found in the inner circle and all were from the day before (except for one whose colour is unknown). The third day only two flea beetles were found, both again in the inner circle. One of them was coloured red, indicating that it was released the day before, the colour of the other flea beetle was unknown. The last day six flea beetles were found. Two of them were in the middle circle (B-circle in figure 9), the others again in the inner circle. One of the flea beetles in the middle circle was unmarked, while the other was red. In part 14 of the inner circle two flea beetles were found of which one was unmarked, the colour of the other flea beetle in this section was unknown. The remaining flea beetles were coloured red, meaning they were released on the second day.

While changing the *R. sativus* plants for *B. nigra*, the following sections contained a flea beetle: A12, C9, C11 and C14 (table 13).
The flea beetles found the last day are found in sections A5, B8, B20, B31, C7, C10 and C14 as indicated in table 13.

Table 12, the number of flea beetles found in the parts on different times with their colour. The first and the seventh column give the day of the experiment. The second and the eight column give the time at which the monitoring of a single part of one of the three circles started. The third, fifth, ninth and eleventh column tell which parts of the segment are watched at that time an the fourth, sixth, tenth and twelfth column give the number of flea beetles found from each colour.

Day1	time	part	Found flea beetles	part	Found flea beetles	Day3	time	part	Found flea beetles	part	Found flea beetles
	0:00	C13	0	B2	0		0:00	B14	0	C1	0
	0:10	C12	0	C5	0		0:10	B2	0	B1	0
	0:20	C28	0	C6	0		0:20	C12	0	C42	0
	0:30	B4	0	A13	0		0:30	B29	0	B1	0
	0:40	C20	0	A2	0		0:40	B11	0	A6	0
	0:50	C28	0	C7	0		0:50	B5	0	A8	0
	1:00	B21	0	B3	0		1:00	B17	0	B3	0
	1:10	B22	0	C11	0		1:10	B31	0	A13	1 red
	1:20	A11	0	A9	0		1:20	A2	0	C14	0
	1:30	C19	0	A9	0		1:30	C2	0	A11	0
	1:40	B21	0	A9	0		1:40	B15	0	B4	0
	1:50	B1	0	B24	0		1:50	C37	0	B12	0
	2:00	A14	0	B4	0		2:00	B22	0	A8	1 unknown
	2:10	A15	0	B6	0		2:10	A10	0	B11	0
							2:20	C36	0	C31	0
							2:30	B30	0	B4	0
							2:40	A7	0	B18	0
							2:50	B3	0	B27	0
							3:00	C20	0	A10	0
							3:10	A6	0	A1	0
							3:20	A7	0	B1	0
							3:30	B13	0	C15	0
Day2	time	part	Found flea beetles	part	Found flea beetles	Day4	time	part	Found flea beetles	part	Found flea beetles
	0:00	C21	0	C7	0		0:00	C17	0	B2	0
	0:10	B30	0	B12	0		0:10	B10	0	C25	0
	0:20	C45	0	A14	1 unmarked		0:20	B11	1 red	C13	0
	0:30	C14	0	B31	0		0:30	C1	0	A7	0
	0:40	B31	0	C35	0		0:40	C44	0	B2	0
	0:50	C40	0	C19	0		0:50	C9	0	B21	0
	1:00	B6	0	A6	1 unmarked		1:00	C24	0	B28	0
	1:10	C23	0	B29	0		1:10	C41	0	A10	0
	1:20	B12	0	C46	0		1:20	A16	1 red	A6	0
	1:30	C9	0	C1	0		1:30	C45	0	A8	0
	1:40	A2	0	A13	0		1:40	B21	0	B24	1 unmarked
	1:50	A15	0	B23	0		1:50	B23	0	A10	0
	2:00	A16	0	C44	0		2:00	C41	0	B10	0
	2:10	A6	1 unmarked	A10	1 unknown		2:10	A7	0	B24	0
	2:20	A2	0	C44	0		2:20	A3	1 red	B8	0
	2:30	A6	1 unmarked	B15	0		2:30	B7	0	C39	0
	2:40	B28	0	C9	0		2:40	C12	0	B19	0
	2:50	B24	0	A16	0		2:50	B9	0	C2	0
	3:00	A5	0	C35	0		3:00	A13	0	C33	0
	3:10	C11	0	B29	0		3:10	B6	0	B27	0
	3:20	C19	0	A13	0		3:20	A9	0	C4	0
	3:30	B12	0	A3	0		3:30	A14	1 unmarked, 1 unknown	C2	0
	3:40	B27	0	B10	0						
	3:50	A10	0	A7	1 unmarked						

Table 13 the colour and the number of flea beetles found in different parts of the three circles. The top row states the reason for the checking of flea beetles. Switching *R. sativus* for *B. nigra* was done on the morning of the third day, before the experiment actually begun. The collection of *B. nigra* was done on the fourth day after the flea beetle monitoring was entirely finished. Below that first row the first and third column state the parts in which flea beetles have been found. The second and fourth column give the number of flea beetles from each colour.

Switching *R. sativus* for *B. nigra*		Collecting *B.nigra*	
Part	Found flea beetles	Part	Found flea beetles
A12	1 unmarked	A5	1 orange
C9	1 red	A14	1 unknown and 1 unmarked
C11	1 unmarked	B8	1 unmarked
C14	1 unmarked	B20	1 unmarked
		B31	1 unknown
		C7	1 unmarked
		C10	1 unknown
		C14	1 unmarked

Conclusion & Discussion

In the experiment only very few flea beetles were found on the circles, this can partly be explained because the three circles contained only a very small part of the entire field and the segments of the circles which were checked were only a small part of these circles. Another explanation could be the weather was not optimal for flea beetle migration. This is supported by the fact that after a rainy day many flea beetles were still in the jars at the release side when we were making ready to leave. The fact that the flea beetles which were released were never found again the same day might indicate that there was not enough time for the flea beetles to find the plants in one day.
Because so few flea beetles were captured in the field, it is difficult to draw any conclusions from the experiment.
It is nice to see that the flea beetles which were found in a part of a circle were found there still one and a half hour later (day 2, part A6). The next day the flea beetle had disappeared from this place. This might indicate that flea beetles stay in a place for a while if there is a host plant, but also that they will not stay there for an extended period. It could be that the flea beetle wanted to find a better host plant or that it wanted to find a mate.
During the experiment most of the flea beetles which were found were unmarked, this might have more than one reason. One of the reasons is that the unmarked flea beetles were released first and therefore had more time to spread over the area and find the host plants. The second reason is that it might be that some of the unmarked flea beetles found in the end were marked but lost their colour. In a previous experiment (Appendix 3) it was shown that it is difficult to mark flea beetles for a longer period. The third reason is that there were already flea beetles in the area, which have been caught and since they were not marked they were mistaken for flea beetles released on the first day.

Recommendations:
If an experiment similar to this one is done again, it would be better to do it in better weather conditions, more sun, less rain and a little warmer. The experiment may give better results when the circles are made smaller, this way it is more probable that also the first day flea

beetles will be found. To get higher numbers of found flea beetles the number of released flea beetles could also be raised.
An area should be found in which it is certain that there are no flea beetles, this way they cannot influence the data.
Instead of marking the flea beetles it would also be an option to use a different area for each release, the disadvantage that has is that it will take more time, since only one release can be monitored at a time. Also more approximately the same areas must be found of which one can be sure that there are no flea beetles.
A nice thing to investigate in the future may be whether there is a difference in dispersal between males and females.

Appendix 6 Two choice experiment

This experiment was done after returning from Denmark where a mark recapture experiment would be performed which did not work. The experiment which was performed in the end was not yet known when this short experiment took place.

Objective: To test whether susceptible larvae of *P. nemorum* can detect leaves from different plant species and if they show a preference to start feeding on *R. sativus* leaves rather than on *B. vulgaris* leaves.

Material and methods:
In this experiment small petridishes, with a diameter of 8.8 cm, were used. A piece of filter paper was cut so it fitted exactly in the petridish. After moistening, the filter paper was put in the petridish. On top of the filter paper a leaf disc was put about half a centimetre from the edge of the petridish. In case two different leaf discs were used they were put about half a centimetre form the edge on opposite sides. The chosen leaves were healthy and not very young but young enough to still be growing, the cotyledons were not used. The used combinations were: *R. sativus* on one half and nothing on the other, *B. vulgaris* on one half and nothing on the other and *R. sativus* on one half and *B. vulgaris* on the other. These leaf combinations were used because the susceptible larvae should be unable to feed on *B. vulgaris*, while also flea beetles exist which can feed on this plant. The *R. sativus* is a good host plant for *P. nemorum* and therefore this plant can be used to test whether the larvae can find the leaves.
After this a larva of susceptible *P. nemorum* was put in the middle of the Petri dish. Then the lid was put on top. The larvae were active and moving when they were taken for the experiment and had never been fed. The larvae were checked three times on following days starting the day after making the set-up. It was written down whether the larvae were still alive, on which side they were and whether they were in the leaf. Every combination has been tested with ten different larvae.

Results:
When *R. sativus* was tested against nothing, three of the larvae found a leaf disc within one day and started to mine it, they were still mining it when the experiment ended (table 14). Two of the larvae were lost after one day. The other five larvae died within one day. In the test of *B. vulgaris* against nothing, two of the larvae were also lost after one day, one was however found again on the second day. One of the larvae was sitting on the leaf disc when checked the first day, two others were also on the *B. vulgaris* side, but not on the leaf disc. Five of the larvae had already died before the first check, four of them were on the side with the *B. vulgaris* leaf of which one was actually on the leaf disc. The second day all larvae were dead, of the two which had not died before, one died in between the two halves and the other on the leaf disc. When testing the two different plant species together, one was lost after one day, one was on the *R. sativus* leaf disc and two were mining it. The other six larvae were all dead on the first day. Four of them died *on B. vulgaris* leaf disc, one in the middle and one on the *R. sativus* leaf disc. The larva which was on the *R. sativus* leaf disc the first day started to mine it before the second check. The three larvae alive on the last day, were all mining the *R. sativus* leaf discs from day one or day two till the end.

Table 14, the situation of the larvae on three following days in the two choice experiment. The first column states the test the larvae were in. The second column is the number of the larvae, the third column states the place and condition the larvae were in on the first day, the fourth column states the place and condition the larvae were in on the second day and the last column states the place and condition the larvae were in on the third and last day.

	larva	day 1	day 2	day 3
R. sativus and nothing	1	in leaf	in leaf	in leaf
	2	in leaf	in leaf	in leaf
	3	in leaf	in leaf	in leaf
	4	lost	lost	lost
	5	lost	lost	lost
	6	dead in middle		
	7	dead in middle		
	8	dead in middle		
	9	dead on lid		
	10	dead somewhere, place unknown		
B. vulgaris and nothing	1	dead on Barbarea		
	2	dead on Barbarea		
	3	dead on Barbarea side		
	4	dead on Barbarea side		
	5	dead opposite of Barbarea		
	6	lost	lost	lost
	7	lost	dead opposite of Barbarea	
	8	on Barbarea	dead on Barbarea	
	9	on Barbarea side	dead in middle	
	10	on lid on Barbarea side	dead on lid Barbarea side	
R. sativus and *B. vulgaris*	1	lost	lost	lost
	2	on radish leaf	in radish leaf	in radish leaf
	3	in radish leaf	in radish leaf	in radish leaf
	4	in radish leaf	in radish leaf	in radish leaf
	5	dead on Barbarea leaf		
	6	dead on Barbarea leaf		
	7	dead on Barbarea leaf		
	8	dead on Barbarea leaf		
	9	dead on radish leaf		
	10	dead in middle		

Conclusion and Discussion:

The larvae which were lost already at the first day could have escaped by sliding out between the petridish and its lid. The larvae which only had access to *B. vulgaris* all died before the end of the experiment, this indicates that the larvae could indeed not feed on this plant. Half of the larvae were dead on the first day of all set-ups, this might indicate that the larvae were not in a very good condition and therefore died before they could start mining a leaf. In the experiment using only *R. sativus*, the dead larvae were mostly found in the middle, this support the idea that they might not have been able to find the leaf discs because of a poor condition. The larvae that were still alive were all able to find the leaf discs and no larva died on the opposite site, this might indicate that the larvae were able to detect the leaf discs and moved towards it when they were fit enough. Another possibility is that the three larvae that managed to find the leaf discs were in such a good shape that they managed to move over the entire petridish until they had found a leaf to mine. This is unlikely however, because then the possibility that one of the larvae had died on the opposite side would have been quite large.

In the experiment using only B. *vulgaris* it is remarkable to see that most of the larvae did die on the side of the leaf disc, only two larvae died on the other side and one died in the middle. This indicates that the larvae were indeed attracted to the leaf discs although they were unable to feed from it. Three larvae actually died on the leaf disc. This could be because they tried to feed on it and died because of toxic components. Another possibility is that they detected the leaf disc and died while they were looking for something they could eat. The flea beetle larva which died in the middle had been seen at the *B. vulgaris* side the day before. This might indicate that the larva first detected the leaf, tried to find something edible and then decided that he would have to look elsewhere to find a suitable leaf.

In the experiment using both *R. sativus* and *B. vulgaris* an equal number of larvae ended up on both sides. This indicates that the larvae did not have any preference for any of the two species before eating them. Since all the larvae that chose the *B. vulgaris* leaf died, it is likely that they tasted the leaf and died from a toxic component. If they did not eat it because they did not like it, they could have started moving again after which they might have found the *R. sativus*.

To draw good conclusions the experiment should be repeated with more larvae, since only thirty were used in this experiment, four of them were lost and half of them died before the first check. It might also be better to check the larvae earlier, this way the chance that half of the larvae are dead at the first check up is smaller. To make sure no larvae can get lost it might be a possibility to seal the closed lid, for example with parafilm.