# The role of enemy-free space in the host range expansion of the flea beetle *Phyllotreta nemorum*.

**Niels Kerstes**
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**Author:**

Niels A. G. Kerstes
Rouwenhofstraat 26
6701 BB Wageningen
The Netherlands

niels.kerstes@wur.nl

**Supervisors:**

Dr. Jens Kvist Nielsen
Institut for Grundvidenskab/Bioorganisk kemi, Den Kgl. Veterinær- og Landbohøjskole
Thorvaldsensvej 40, 6. sal
Room T651
1871 Frederiksberg C
Denmark

jeni@kvl.dk

Dr. Peter W. de Jong
Laboratorium voor Entomologie, Wageningen Universiteit
Binnenhaven 7
Room 028
6709 PD Wageningen
The Netherlands

peter.dejong@wur.nl

## Preface and acknowledgement

Doing a Master Thesis is a compulsory part of the Master Biology program at Wageningen University. As the subject of this Thesis I chose a tritrophic interaction between a phytophagous insect, its host plants and its parasitoids. This subject interests me because it deals with both evolutionary ecology and, indirectly, biological control. Furthermore I think insects are convenient research objects, because of their small size, short lifespan and high abundance. During my study Biology I learned about the large impact insects have on ecosystems and human beings.

As an Ecology student I wanted to have done real field work at least once in my study. Furthermore I also wanted to go abroad, to experience how it is to work in a strange environment. All these aspects were combined in this research.

I would like to thank my two supervisors, Dr. Jens Kvist Nielsen and Dr. Peter de Jong, for their excellent help and support during this Thesis. Furthermore I would like to thank Yde Jongema, Dr. Niels Agerbirk and Dr. Thure Pavlo Hauser for sharing their knowledge and expertise with me. I am also grateful to Maja Rohr Hansen for rearing and maintaining the insects and plants in the laboratory. Special thanks go out to Jens Vinther Frederiksen, who was so kind to let me live in his house during my stay in Denmark.

Doing this Thesis gave me a lot of insight into how to set up and carry out an ecological research. It taught me that especially flexibility is an important characteristic of a good researcher. I really enjoyed both doing the research and living in Copenhagen.

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# Contents

## Abstract

The mortality of phytophagous insects caused by natural enemies can differ between plant species. This difference can influence the host plant choice of the insects. If a certain plant species gives the insects a higher degree of protection against natural enemies than an alternative host plant, then this plant species is said to provide enemy-free space to the insects.

The larvae of the oligophagous insect *Phyllotreta nemorum* L., a flea beetle, are known to feed on a limited range of crucifers. In East Denmark a population was discovered in which large numbers of *P. nemorum* larvae use *B. vulgaris* ssp. *arcuata* (Opiz.) Simkovics as a host plant, while this plant species was thought to be a very unlikely host plant. Compared to other host plants, *B. vulgaris* seems a less profitable host plant for larvae of *P. nemorum*. In this research it was investigated, by means of field work and laboratory work, whether the concept of enemy-free space could explain why *P. nemorum* started to use *B. vulgaris* as a host plant.

The results of this study indicate that *B. vulgaris* provides enemy-free space to larvae of *P. nemorum* in two different ways. It was found that the flea beetle larvae feeding on their most abundant host in East Denmark, *Sinapis arvensis* L. (Cruciferae), were parasitized by the parasitoid wasp *Aneuclis brevicauda* (Thomson) (Hymenoptera: Ichneumonidae), while the flea beetle larvae feeding on *B. vulgaris* were not. Furthermore it was found that *B. vulgaris* grows and develops earlier in the season than *S. arvensis*, with the consequence that flea beetle larvae were able to peak earlier on *B. vulgaris*. This enabled them to escape from extremely high parasitism levels at the end of the season, caused by the parasitoid wasp *Diospilus morosus* (Hymenoptera: Braconidae). Looking at the whole season, this resulted in a higher parasitism rate of flea beetle larvae by *D. morosus* on *S. arvensis* than on *B. vulgaris*. This is an interesting finding, because if you look at each separate collection date, percentage of parasitism was never significantly lower on *B. vulgaris* than on *S. arvensis*.

This study shows the importance of within-season variation of parasitism rate, and the importance of having data about the proportion of the population of host insects present at the time of measuring parasitism rate. Possible explanations for the absence of parasitism by *A. brevicauda* on *B. vulgaris*, and ways to investigate them, are given.

# 1. Introduction

## 1.1. Enemy free space

It is well known that many phytophagous insects are, more or less, specialized in their use of host plants (Ehrlich and Murphy 1988, Stamp 2001). There are several reasons, both non-ecological and ecological, that can explain the high level of specialization of these insects. A non-ecological reason might be that it is impossible for the insects to simultaneously maximize performance on host plants with different defensive compounds (Ehrlich and Murphy 1988, Keese 1997). This has to do with the costs of maintaining detoxification enzymes (Keese 1997). Related species of butterflies often use plants that are chemically similar. Furthermore, introduced plants are mostly colonized by insects that feed on chemically similar plants (Jaenike 1990). However, also ecological factors could play an important role in host specialization. The ability to locate mates, differences between geographic regions in the abundance of host plants, feeding mode, synchronization of herbivore life history with plant phenology and predation and parasitism are all examples of factors that could play a role in host plant specialization (Keese 1997).

One would expect that the specialized phytophagous insects use the host plants on which their performance is optimal. Some herbivores indeed show a positive correlation between oviposition preference and larval performance (Denno et al. 1990). However, it is often found that herbivorous insects use plants or plant parts on which larval performance is sub-optimal (Gratton and Welter 1999, Mulatu et al. 2004). An explanation for this phenomenon might be that the insects on these apparently sub-optimal host plants are able to escape from competitors, predators, or parasitoids (Mulatu et al. 2004). Enemy attack is the most frequent cause of death for immature insect herbivores (Hawkins et al. 1997), so indeed the occurrence of natural enemies might have a large effect on host plant choice.

If a plant species gives the insect a higher degree of protection against natural enemies than an alternative host plant, then this plant species is said to provide enemy-free space to the insect (Ballabeni et al. 2001). Another definition of enemy-free space is ‘ways of living that reduce or eliminate a species’ vulnerability to one or more species of natural enemies’ (Berdegue et al. 1996). Dozens of examples of researches involving enemy-free space can be found in the literature (Berdegue et al. 1996, Scheirs and De Bruyn 2002).

There are several mechanisms explaining how enemy-free space can exist. It is possible that herbivorous insects use plant secondary compounds to defend themselves against their natural enemies (Keese 1997). An example of this is given by Denno et al. (1990). The willow beetle *Phatora vitellinae* L. secretes plant-derived chemicals as a defence against predators. This beetle has a preference for plants containing high levels of the secondary compounds used by the beetle to protect itself.

A lot of predators and parasitoids use visual or chemical cues to locate their prey or hosts (Vet and Dicke 1992, Keese 1997). If herbivores shift to a new host plant that is not easily, or not at all, detected by their natural enemies they might escape from these enemies, resulting in a lower mortality caused by predation and parasitism (Keese 1997). It is also found that enemy free space can arise in case there is an asynchrony in seasonal distribution of natural enemies and their hosts (Feder 1995). Larvae of the apple maggot fly *Rhagoletis pomonella* (Diptera: Tephritidae) feeding on apples suffered from lower mortality by parasitoids than larvae of this species feeding on hawthorn. One reason for this difference was that the apples had an earlier fruiting phenology than the hawthorns (Feder 1995, Feder and

Filchak 1999). This enabled the larvae of the apple maggot fly to develop earlier in the season, when the populations of natural enemies are still relatively low (Feder 1995).

## 1.2. Case study in Denmark

In this research the tritrophic interaction between a phytophagous insect, its host plants and its natural enemies was investigated. This interaction was studied in several locations in East-Denmark, during the spring and summer of 2006.

The phytophagous insect involved in this study is *Phyllotreta nemorum L.* (Coleoptera: Chrysomelidae: Alticinae), also known as the large striped flea beetle (Alford 2003). *P. nemorum* has only one generation per year, and adults overwinter in diapause in the soil. Eggs are laid in the soil close to the host plants, and newborn larvae have to climb the plant to find a suitable feeding site (Nielsen 1997). The larvae of this oligophagous insect are leafminers on a limited range of crucifers (Nielsen 1997, de Jong and Nielsen 1999). Examples of suitable host plants are *Sinapis arvensis* L., *Raphanus sativus* L. and *Cardaria draba* (L.) Desv. (de Jong and Nielsen 2002). The plant genus *Barbarea* also contains several species which are suitable host plants for *P. nemorum*. However, one species of this genus, *Barbarea vulgaris* R.Br., was thought to be an unlikely host plant. In laboratory bioassays it showed very variable suitability for larval development of *P. nemorum* (de Jong and Nielsen 2002).

In Denmark two subspecies of *B. vulgaris* are present: *B. vulgaris* ssp. *arcuata* (Opiz.) Simkovics and *B. vulgaris* ssp. *vulgaris*. The latter is uncommon in Denmark (de Jong and Nielsen 2002). Two types of *B. vulgaris* ssp. *arcuata* are distinguished, based on morphological and chemical differences. The 'P' type, with pubescent leaves, is a suitable host for flea beetles during the whole year. The 'G' type, with glabrous leaves, is normally an unsuitable host for flea beetle larvae during the summer (Agerbirk et al. 2001). However, certain genotypes of *P. nemorum* recently found in Denmark are able to use this 'G' type plant during the whole season, even during the summer (Nielsen 1997, Agerbirk et al. 2001). It seems that these flea beetle larvae have found a way to deal with the defence mechanisms of the *Barbarea* 'G' type (de Jong and Nielsen 2002, Renwick 2002). From now on in this report the 'G' type of *B. vulgaris* ssp. *arcuata* (Opiz) Simcovics will be simply called *Barbarea*, because this is the plant of interest of this research.

Compared to *R. sativus* and *S. arvensis*, *Barbarea* seems a less profitable host plant for larvae of *P. nemorum*. Development time on *Barbarea* is longer than on the other host plants (Nielsen 1999). Furthermore it is often found that resistance is associated with negative pleiotropic effects, which reduce the fitness of resistant individuals on other plants compared to susceptible individuals (de Jong and Nielsen 2002). Yet, resistance against the defence mechanisms of *Barbarea* has developed. Because of the costs associated with the resistance, it is expected that there is another mechanism involved which made it beneficial for the flea beetles to start using *Barbarea* as a host plant. The positive effect of this mechanism on the fitness of the flea beetles should be larger than the negative effects associated with the resistance.

The concept of enemy free space may give an explanation for the development of the seemingly illogical resistance. The larvae of *P. nemorum* are known to be attacked by several hymenopterous parasitoids, including the braconid *Diospilus morosus* (Reinhardt), the ichneumonid *Aneuclis brevicauda* (Thomson) and two chalcids *Eulophus* sp. and *Pnigalio soemius* (Walker) (Alford 2003). In the part of Denmark where *Barbarea* resistant flea beetles are found, *A. brevicauda* seems to be one of the most important natural enemies of *P. nemorum*. Parasitism levels of flea beetle larvae by *A. brevicauda* of more than 60% were

found (de Jong and Nielsen unpublished data). If *Barbarea* provides an enemy-free (or enemy-reduced) space for *P. nemorum*, this might explain the observed host shift by the flea beetle and the evolution of the associated resistance to the defence mechanisms of *Barbarea*. Study has already shown that *Barbarea* is not a completely enemy-free space (parasitism on *Barbarea* is not zero), but there is some indication that parasitism of *P. nemorum* by *A. brevicauda* on *Barbarea* is lower than on alternative host plants like *Sinapis arvensis* (de Jong and Nielsen, unpublished data).

*Barbarea* does, most probably, not provide an enemy-free space by either supplying flea beetles with chemical components which they can use as a defence against natural enemies, or by being less detectable or even undetectable for natural enemies. In earlier research it turned out that relatively high percentages of flea beetle larvae feeding on *Barbarea* were parasitized by parasitoid wasps (de Jong and Nielsen unpublished data). The possible reduction of parasitism on *Barbarea* could be explained by the fact that *Barbarea* develops and grows earlier in the season than the alternative host plants. Therefore also the larvae of *P. nemorum* can develop earlier in the season if they feed on *Barbarea*. The life cycles of the natural enemies of *P. nemorum* are possibly adapted to the life cycle of *P. nemorum* feeding on their normal (old) hosts, as was also found in the apple maggot fly example of Feder (1995). Therefore it is possible that at the time the larvae of *P. nemorum* are feeding and growing on *Barbarea* early in the season, only few adults of *A. brevicauda* are present. Therefore larvae of *P. nemorum* feeding early on *Barbarea*, when other host plants are still absent, will be able to escape parasitism, which could compensate for the lower suitability of *Barbarea* as a host plant.

## 1.3. Testing for enemy free space

### *1.3.1. Plant characteristics*

*Sinapis arvensis*, also known as wild mustard, is the most common host plant of flea beetles in East Denmark (J.K. Nielsen personal communication). Therefore in this research this plant species was chosen as the one to represent an old host of flea beetle larvae. *Sinapis arvensis* shall be simply called *Sinapis* in the rest of this report. *Sinapis* is an annual (Warwick et al. 2000), while *Barbarea* can be an annual, a biennial or a perennial (Macdonald and Cavers 1991). The fact that *Barbarea* can overwinter as a rosette, and *Sinapis* can not, could, at least partly, explain why *Barbarea* can be used earlier in the season by flea beetle larvae than *Sinapis*. To confirm that indeed *Barbarea* grows and develops earlier than *Sinapis* in the study areas involved, some phenological characteristics (biomass development, percentage of plants with flowering stalk, percentage of flowering plants) of both plant species were measured.

### *1.3.2. Parasitism rates*

Proving that parasitism on *Barbarea* is less than on *Sinapis* is not sufficient to infer that the host expansion of *P. nemorum* is caused by the existence of an enemy-free space. Low enemy impact could also be the result of low population densities on the new host plant. Also it is not clear that at the time of the host expansion the same level of enemy-induced mortality existed on both new and old host plant (Mulatu et al. 2004), although it seems that the colonization of *Barbarea* by *P. nemorum* occurred recently (de Jong and Nielsen 1999). To really test for

enemy-free space, three hypotheses formulated by Berdegue et al. (1996), should be tested. If a new host plant indeed provides an enemy-free space, then:
1. the fitness of the organism in the original habitat with natural enemies should be less than the fitness of the organism in the original habitat without natural enemies;
2. the fitness of the organism in the alternative habitat with natural enemies should be greater than the fitness of the organism in the original habitat with natural enemies, and;
3. the fitness of the organism in the alternative habitat without natural enemies should be less than the fitness of the organism in the original habitat without natural enemies.

The first hypothesis tests the importance of natural enemies in the system. The second hypothesis tests whether mortality caused by natural enemies is really less on the new host plant. The third hypothesis tests whether there is a cost of feeding on the new host plant. This third condition is necessary to make sure that escape from natural enemies is really the only driving force behind the host switch or extension (Mulatu et al. 2004).

Because relatively high levels of parasitism of *P. nemorum* by *A. brevicauda* (up to 60%) were found on original host plants, one can assume that parasitism indeed reduces fitness of the flea beetles. Therefore, hypothesis 1 is true. As mentioned before, *Barbarea* is found to be a less profitable host plant compared to the alternative host plants. Therefore, also hypothesis 3 is true. To demonstrate that indeed *Barbarea* provides enemy-free space for *P. nemorum*, only hypothesis 2 still has to be confirmed. In this research this second hypothesis was tested, by means of field and laboratory work in East Denmark.

If *Barbarea* provides enemy-free space to *P. nemorum*, and if the idea that this is caused by the opportunity of earlier development of flea beetle larvae on this plant is true, then most probably *Barbarea* will only provide enemy-free space at the beginning of the season. Therefore, one whole *P. nemorum* season was monitored. In case *Barbarea* provides enemy-free space to *P. nemorum* during the whole season, another explanation for the occurrence of enemy-free space has to be found (like Barbarea being less detectable for its natural enemies than other host plants)

### 1.4. Testing for the importance of *A. brevicauda* as natural enemy

Although from earlier research it seems that *A. brevicauda* is the most important natural enemy of *P. nemorum* in East Denmark, this is not completely sure. Therefore all parasitoids reared from *P. nemorum* larvae in this study were identified to species level.

### 1.5. Testing the seasonal variability in defences of *Barbarea*

Defence mechanisms of *Barbarea* against herbivory by flea beetle larvae is found to be variable throughout the season (Nielsen 1997, Agerbirk et al. 2001). Even susceptible larvae of *P. nemorum* are able to feed on *Barbarea* early in the season. This temporal variation in the defences of the plant could have facilitated the host range extension of *P. nemorum*, because susceptible larvae may have managed to complete development on *Barbarea* early in the season before defensive compounds of *Barbarea* were produced in sufficient quantities. Resistance to the defence mechanisms of *Barbarea* could have arisen in these larvae as random mutations (Nielsen 1997). In this research, it was investigated whether really susceptible flea beetle individuals in the field use *Barbarea* as a host plant early in the season.

## 1.6. Timing of the study

*P. nemorum* has only one generation per year. De Jong and Nielsen (unpublished data) performed their research from mid-June to the second week of July, which covered the larger part of the larval period of the flea beetle in the field. Larvae of *P. nemorum* were found to be parasitized by *A. brevicauda* from the end of May to the end of July (Alford 2003). Because it was essential that this study included the start of the flea beetle and parasitoid season, this research was started at the beginning of April and ended at the end of July.

The effect of natural enemies on the population of its host can be very variable throughout the season (Scheirs and De Bruyn 2002). Therefore it is important that the whole larval season is monitored, of course as frequently as possible. In this research it was made sure that indeed the complete larval season was included. In the most accessible study areas larvae were sampled every week.

## 1.7. Study areas

In East Denmark three well studied areas are known where both *Sinapis* and *Barbarea*, resistant flea beetles and *A. brevicauda* are present: Ejby, Kværkeby and Amager. On the fourth location, Suserup, only *Sinapis* was present. Figure 1 shows the location of the four study areas.

In Ejby there was 1 site where *Barbarea* was growing, and 1 site where *Sinapis* was growing. The distance between the two sites was approximately 350 meters. In Kværkeby there were 2 *Barbarea* sites, in this research called Kværkeby 1 and Kværkeby 3, and 1 *Sinapis* site, in this research called Kværkeby *Sinapis*. The distance between Kværkeby 3 and Kværkeby *Sinapis* was approximately 1000 meters, the distance between Kværkeby 1 and Kværkeby *Sinapis* around 1650 meters. In Amager no suitable *Sinapis* site was found, so there was only a *Barbarea* site.

In East Denmark there are no sites where *Barbarea* and *Sinapis* are growing mixed or right next to each other. Therefore it was decided to sow experimental plots with *Barbarea* and *Sinapis* right next to each other. This was done in Kværkeby, in between Kværkeby 3 and Kværkeby *Sinapis*.

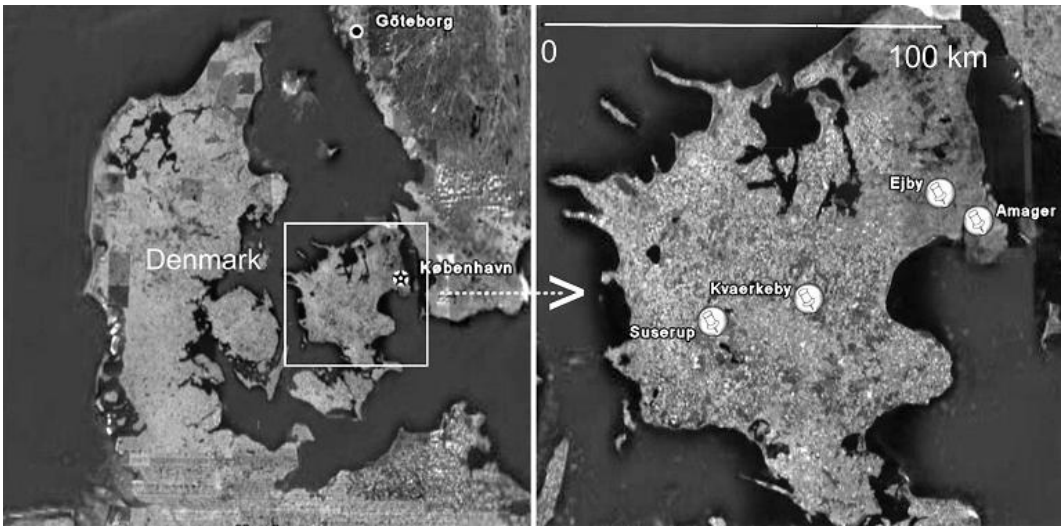

Figure 1. The location of the 4 study areas of this research (Google Earth images).

## 1.8. Research questions

Summarizing, the main question of this research was:
- Does *Barbarea* provide an enemy-free space to *P. nemorum*?

Other questions that were tried to be answered within this research were:
- Does *Barbarea* grow and develop earlier in the season than *Sinapis*?
- Do larvae of *P. nemorum* start to use *Barbarea* as a host plant earlier in the season than they start to use *Sinapis*?
- Do larvae of *P. nemorum* peak earlier in the season on *Barbarea* than on Sinapis?
- Are parasitism levels of *P. nemorum* larvae by *A. brevicauda*, or other parasitoids, on *Barbarea* lower than those on *Sinapis*, and if yes, when?
- Are parasitism levels of flea beetle larvae on *Barbarea* in general lower than those of flea beetle larvae on *Sinapis* during the whole season?
- Is the life cycle of *A. brevicauda*, or other parasitoids involved, adapted to the life cycle of *P. nemorum*?
- Do susceptible *P. nemorum* adults use Barbarea early in the season?

These questions were tried to be answered by:
- collecting adult flea beetles on *Barbarea* early in the season to check if they are really resistant to the defence mechanisms of *Barbarea*;
- measuring phenological characteristics of *Barbarea* and *Sinapis* throughout the whole season;
- measuring parasitism levels of flea beetle larvae on both *Barbarea* and *Sinapis* throughout the whole season;
- identifying the parasitoids found to species level;
- measuring densities of flea beetle larvae on *Barbarea* and *Sinapis* throughout the whole season

# 2. Materials and methods

## 2.1. Testing the seasonal variability in defences of Barbarea

Adult flea beetles were collected in the field from the first moment they were present. The beetles were collected only from *Barbarea*. It was assumed that beetles collected on *Barbarea* were also feeding on this plant. The beetles were collected in Amager, Ejby and Kværkeby (subarea 1 and 3). The first beetles were collected on 20 April 2006, the last ones on 31 May 2006.

The adult beetles were collected by means of an aspirator, and taken to the laboratory in small individual vials. In the laboratory *Barbarea* G-type plants were present which were reared under constant conditions (20ºC), making sure they were always resistant to susceptible flea beetles. It was tested whether or not the flea beetle adults collected from the field could feed on the resistant *Barbarea*, and thus whether or not these beetle adults were susceptible or resistant to the defence mechanisms of *Barbarea*. The sex of all beetles was also determined.

The flea beetles collected were put individually in vials of 185 ml. On the bottom of these vials a moisture disc made of a mixture of gypsum and carbon was placed. The vials were closed with a plastic lid with a central hole, which was closed with a wad of cotton wool. Two round leaf disks (diameter = 1.1 cm) from two different *Barbarea* leaves were placed on the bottom of the vial, on the moisture discs. Only healthy looking Barbarea leaves large enough for at least four leaf disks were used. The leaf disks were kept in place with one needle in the centre of each disk. The flea beetles were individually placed inside the vial, kept in light at a temperature of 22 ± 2 °C, and removed again after 3 days. The leaf disks were then checked for damage caused by feeding of the flea beetle adults. A beetle was said to be a feeder if it had eaten as much as or more than the chosen threshold of 20 $mm^2$.

At the end of April and the beginning of May the *Barbarea* plants grown in the lab were heavily attacked by aphids. This might have had an effect on the health and thus the resistance of the plant. By this time two beetles from the susceptible line in the laboratory were tested the same way the beetles collected in the field were tested for resistance. It turned out that these two susceptible beetles almost ate as much as 20 $mm^2$, the threshold for a feeder. On 9 May 2006 the aphid population seemed to be under control, thanks to ladybirds and parasitoid wasps, and all bioassays done before this date were done again.

All possible male non-feeders found (except the one from Kværkeby 3, collected 20-04) were crossed with susceptible females from the isogenic line in the lab. The purpose of this cross was to check whether the non-feeders were really susceptible flea beetles. The crossing was done by putting the collected male beetle together with a susceptible female in a 185 ml vial with a moisture disc. Some radish seedlings were added for food, and replaced every three days. Once larvae were visible in the vials, they were transferred to smaller vials with a G-type *Barbarea* leaf. Up to 5 larvae were put together in 1 vial. Also added to the smaller vials was a moist piece of filter paper, to keep the leaf fresh. Larvae were transferred from the 185 ml vials to the smaller vials by using a moist paint brush. After three days it was checked whether the larvae were still alive and feeding on the G-type *Barbarea* leave. If not, they were assumed to be non-feeders. If all offspring of the cross were non-feeders, then it was safe to say that the collected male was a susceptible beetle. For each possible male non-feeder at least 30 larvae were tested.

## 2.2. Testing for enemy-free space

### *2.2.1. Plant characteristics*

Phenological characteristics of both *Barbarea* and *Sinapis* were measured during the whole season at different locations. *Barbarea* characteristics were measured in Amager, Ejby, Kværkeby 1 and Kværkeby 3. *Sinapis* characteristics were measured in Kværkeby and Suserup. In all areas except the *Sinapis* area in Kværkeby 5 plots were chosen in such a way that the whole study area was represented as well as possible. In these plots the height and the diameter of the plants were measured, and it was noted if the plants had a flowering stalk and if they were flowering. In the case of *Barbarea* it was quite clear when a plant had a flowering stalk, because of the rosette shaped vegetative form of this species. The *Sinapis* plants were said to have a flowering stalk as soon as flower buds were visible. The plants were said to be flowering as soon as the first flower bud had opened.

Height and diameter of the plants were not measured exactly, but classified in 5 different classes. These classes are shown in table 1. The classification was chosen only to save time.

*Table 1. The height and diameter classes used in measuring the plants in the field.*

| *class number* | *height/diameter (cm)* |
|---|---|
| 1 | <=5 |
| 2 | 5.1-10 |
| 3 | 10.1-20 |
| 4 | 20.1-50 |
| 5 | >=50 |

For each height/diameter class combination which was present in the field, per species 5 plants were taken to the laboratory. This was done to get an idea about the average biomass of the plants in the plots. Plants to measure the biomass of the class combinations were collected in the field as soon as a certain height-diameter combination was present. The leaves of these plants were picked and dried in an oven for at least 4 days at 70 °C. The leaves of the plants were dried in paper bags, all leaves of one plant together in one bag. After the drying period the dry weight biomass of the leaves was measured for each plant separately. The average biomass of the five plants in each height-diameter combination was calculated. This average biomass per height-diameter combination is used to calculate the average biomass of the plants in a plot, and the average biomass of the plants at a certain location.

As said before at all locations except the *Sinapis* area in Kværkeby 5 square plots were chosen. These plots were all 1 by 1 meter, except 2 *Barbarea* plots in Kværkeby 1. These two plots were 50 by 50 cm, just because the density of plants was very high in these plots. The plots were marked with wooden sticks, one in each corner of the plot. The number of plants in the plots was not counted. If there were less than or around 25 plants in a plot all plants were measured. If there were much more plants in a plot approximately 25 plants were chosen randomly.

The number of plants in the plots was not counted, because it seemed quite impossible anyway to give an indication of the total number of plants at a certain location. This is caused by the very uneven, patchy distribution of the plants. Because it can be quite interesting to know the total biomass of food present at a certain location, I spoke to a plant ecologist at KVL, Dr. Thure Pavlo Hauser, about a way to estimate the total plant biomass in a certain area. However, the suggestion he gave was much too laborious and time consuming. Because nobody had a better suggestion, I dropped the idea to estimate total biomass at the different locations.

The reason no plots were made in the *Sinapis* area in Kværkeby was that in this area it was not allowed to put sticks in the ground. The owners of the area were thinking about ploughing the area (which they by the way never did). Because there was only one other *Sinapis* location, I still decided to measure plant characteristics in this area. While walking through the area, each time 50 plants were chosen as randomly as possible. These 50 plants were measured.

Certain areas at the study locations were soil-treated at different moments before the start of this research. Table 2 shows when the areas in which the different plots were had their last soil treatment, along with some other relevant information. With "soil treatment" ploughing is meant.

Table 2. Information about the last soil treatment in the plots used for measuring plant characteristics.

| *Barbarea* | | |
|---|---|---|
| Amager | | |
| *plot nr* | *last soil treatment* | *extra information* |
| 1 | - | |
| 2 | - | |
| 3 | - | |
| 4 | - | other side road, higher vegetation |
| 5 | - | |
| Ejby | | |
| *plot nr* | *last soil treatment* | *extra information* |
| 1 | - | outside fenced area |
| 2 | 2005 | inside fenced area |
| 3 | 2004 | inside fenced area |
| 4 | - | outside fenced area |
| 5 | 2004 | inside fenced area |
| Kvaerkeby 1 | | |
| *plot nr* | *last soil treatment* | *extra information* |
| 1 | - | |
| 2 | 2005 | 50x50 cm |
| 3 | 2005 | 50x50 cm |
| 4 | 2004 | |
| 5 | 2004 | |
| Kvaerkeby 3 | | |
| *plot nr* | *last soil treatment* | *extra information* |
| 1 | 2004 | |
| 2 | 2005 | |
| 3 | 2003 | |
| 4 | 2005 | |
| 5 | 2004 | |

| *Sinapis*<br><br>Suserup | | |
|---|---|---|
| *plot nr* | *last soil treatment* | *extra information* |
| 1 | 2005 | |
| 2 | 2005 | |
| 3 | 2004 | |
| 4 | 2006 | |
| 5 | 2006 | |

### *2.2.2. Parasitism rate*

To determine the parasitism rates of flea beetle larvae on the two different plants species, leaves containing larvae were picked in the field. This was done at several locations, during the whole larval season. Where and when leaves containing larvae were collected is shown in table 3.

Table 3. Schematic overview of all collection areas and dates concerning larvae collections.

| *location* | *plant species* | *collection dates* |
|---|---|---|
| Amager Ørestad | *Sinapis* | 24 June, 12 July |
| Ejby | *Barbarea* | 9 June, 15 June, 23 June, 30 June, 7 July |
| Ejby | *Sinapis* | 9 June, 15 June, 23 June, 30 June, 7 July |
| Kværkeby | *Sinapis* | 20 June, 12 July |
| Kværkeby 1 | *Barbarea* | 20 June, 12 July |
| Kværkeby 3 | *Barbarea* | 20 June, 12 July |
| Suserup | *Sinapis* | 20 June, 12 July |

Figure 3 shows that Ejby is the only location where the whole larval season was monitored weekly. In Ejby also larvae were collected 1 June, but the larvae were too small to be included into the research. 16 July it was also tried to collect larvae in Ejby, but numbers of larvae were too low. This shows that at this location really the entire season was monitored.

On all dates at all locations it was tried to collect approximately 100 leaves containing large flea beetle larvae. The leaves were put together in plastic bags, keeping the samples collected at different host plants, different locations and collection dates separate. All leaves were kept at a temperature of around 20-28ºC. Old and deteriorating leaves without larvae were removed from the bags. Bags containing *Barbarea* leaves were supplied with extra *Barbarea* G-type leaves as extra food for the larvae if necessary. Bags containing *Sinapis* leaves were supplied with extra *Barbarea* P-type leaves if necessary.

Final instar larvae exit the leaf mines in search for a place to pupate. All leaves were checked once or twice a day for such final instar larvae. These larvae were picked up with a small moist piece of filter paper at the end of tweezers and transferred to a jar, where they could pupate. The jar contained a layer of a mixture of moist peat and medium grain vermiculite. Again larvae from different host plants, locations and collection dates were kept separate. The jars were kept under the same conditions as the bags with leaves.

When larvae were transferred to the jars where they could pupate they were counted. In the jars a leaf was added (*Barbarea* G-type for larvae from *Barbarea*, *Barbarea* P-type for larvae from *Sinapis*) in case some of the final instar larvae still needed some additional

feeding before pupation. This leaf was removed after all larvae had buried into the peat/vermiculite layer to pupate. The jars were then monitored at least twice a day to check whether flea beetles and/or parasitoids had emerged. These beetles and parasitoids were removed and counted. All beetles were killed in a freezer or sent to the Netherlands. Most parasitoids were transferred to cages for later experiments. Some parasitoids were put in 70% alcohol for later identification. Jars were monitored until no more flea beetle adults and/or parasitoids emerged for at least two weeks. Parasitism rates per plants species, per location, per collection period were calculated as the total number of parasitoids of that sample divided by the total number of parasitoids and flea beetles of that sample. Mortality was calculated as 1 minus the total number of parasitoids and flea beetles of that sample divided by the total number of larvae of that sample. It was assumed that mortality of non-parasitized and parasitized beetles was equal, and therefore that the percentage of parasitism amongst dead larvae was equal to the percentage of parasitism of larvae from which a beetle or a parasitoid was reared.

Final instar larvae from all locations were collected from the bags with leaves until the third day after collection. In this way only larvae which were already final (third) instar larvae in the field were included in the analyses. Only in Ejby larvae were collected from the bags until the sixth day after collection. In this way also data about parasitism of the second instar larvae was collected. Larvae collected from the first to the third day were kept together, as well as the larvae from the fourth to the sixth day. In all cases it was tried to put only as many as 100 larvae in one pupation jar.

Only in Ejby every time the exact number of leaves collected was counted. It was tried to collect 100 larvae-containing leaves per plant species per collection date. If this was not possible, it was tried to collect as many leaves with larvae as possible. The data about the total number of leaves collected was used to calculate the density of third and second instar larvae in the field. This is done by dividing the total number of second or third instar larvae by the total number of leaves collected.

### *2.2.3. Experimental plots*

On 19 April 2006 experimental plots were made in Kværkeby. In was chosen to make the plots inside an area which was soil-treated one year earlier. Plot size was 2x2 meters, and the space between plots was also 2 meters. The plots were cleared of most plants and old flowering stalks, so what was left was mostly bare soil. We made two rows of 12 plots, so there were 12 pairs of plots.

Inside each plot either *Barbarea* or *Sinapis* was sown. The seeds used were collected in Kværkeby, the *Sinapis* seeds on 11-09-2003 and the *Barbarea* seeds on 19-08-2004. In each *Barbarea* plot 700 seeds were sown, in each *Sinapis* plot 500 seeds were sown. This is because the *Sinapis* seeds are slightly larger, and therefore might have a higher chance of germinating.

Plant species were assigned randomly to each plot, with both species present in each pair of plots (a coin was used to decide which species was sown in each of the two plots in a pair). Figure 2 shows the experimental design. Seeds were sown evenly distributed in the plots. After sowing the seeds were covered by a small layer of soil, by raking the soil very superficially.

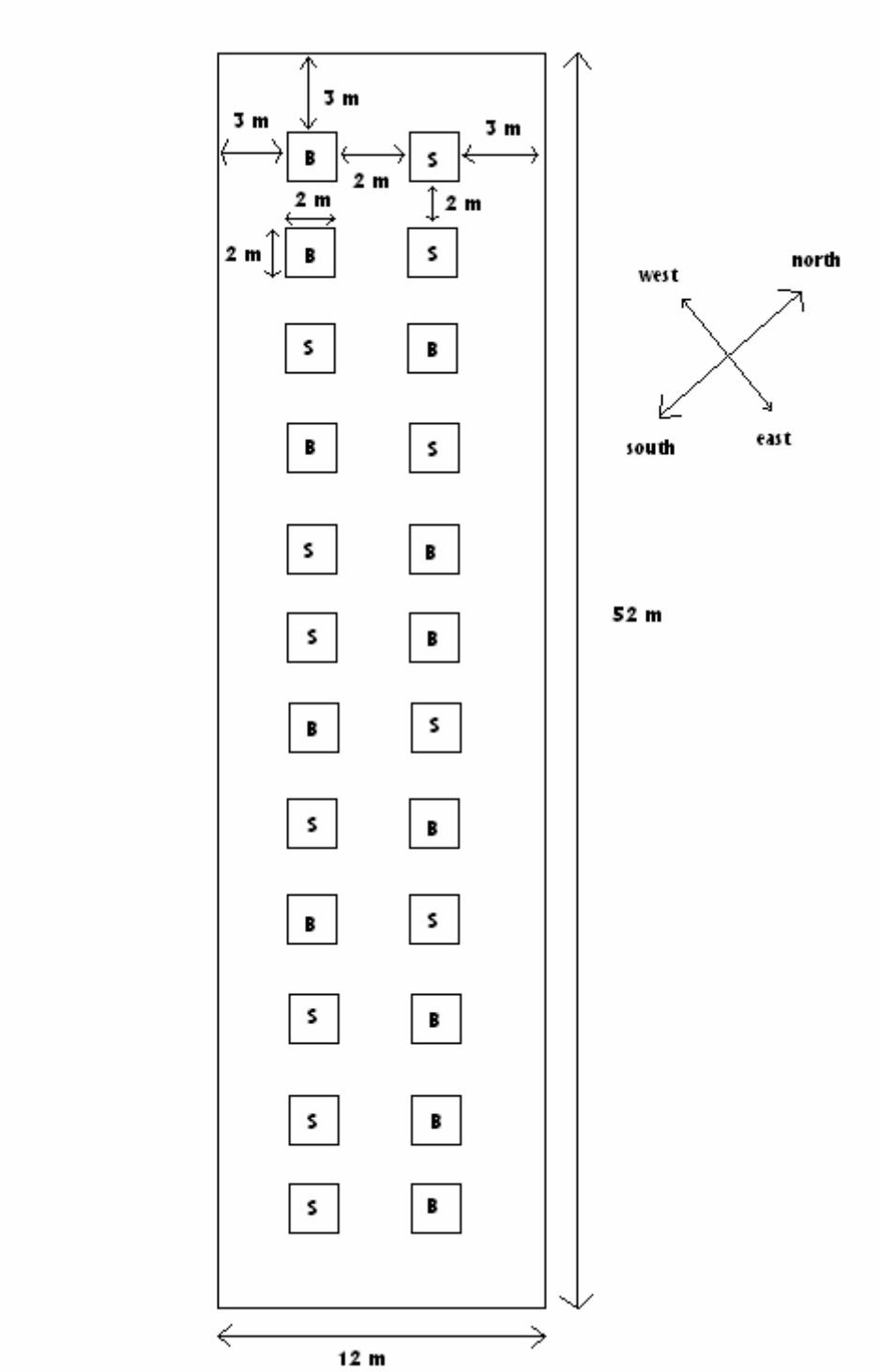


*Figure 2. The arrangement of the plots and the plant species per plot (B* = Barbarea*, S* = Sinapis*).*

Each time we were in Kværkeby after the date of sowing, the plots were cleared of the most persistent weeds. This was done to improve the survival chances of the *Barbarea* and *Sinapis* seedlings.

It was found that the sowing of the *Sinapis* seeds was quite successful. However, the *Barbarea* seeds had not germinated well. Therefore it was decided to plant *Barbarea* seedlings from the laboratory in the *Barbarea* plots. This was done on 6 June 2006, with seedlings which were sown 11 days before. The seedlings were planted inside the plots in turf pots, around 10 pots per plot.

Unfortunately this also did not work, because it turned out that the next time we were in Kværkeby most pots were taken out of the soil, probably by some animals (e.g. geese). Only in one plot still some pots were present, and they seemed to grow quite successfully.

Although the experimental set up did not work out perfectly, it was still possible to get some data out of it. On 12 July 2006 larvae were collected in both the *Sinapis* and the *Barbarea* plot, to compare parasitism rate in these plots.

## 2.3. Testing for the importance of *A. brevicauda* as natural enemy

### *2.3.1. Wasp species identification*

Every time new parasitoid wasps emerged from the larvae collected in the field, they were closely studied. The first parasitoid wasps which emerged were called species A. Most parasitoids found were thought to be of this species. However, one species obviously looked different than species A. This species also needed more days for its development, and behaved differently. They were much more active than species A. This species was therefore called species B. In the end of the season parasitoids emerged which looked and behaved similarly to species A. However, it seemed that the peak of the population of species A was already over, and now these wasps emerged in very high frequencies. This could be explained by assuming that this was either a new generation of species A, or yet a new species. Because this was not certain, these wasps were said to belong to species C.

Samples of species A (from 15 June 2006), species B (from 30 June 2006) and species C (from 7 July 2006) were taken to Wageningen University in 70% alcohol. In Wageningen Yde Jongema identified the wasps to species level. It became apparent that species A and species C were the same. Therefore, in the rest of this report, the results of species A and species C are grouped under the name species A.

### *2.3.2. Rearing the wasps*

As said some of the wasps were put into alcohol for later identification. However, most wasps were put into plastic cages (50 x 30 x 30 cm). At the top of the cages was a lid, and the two long sides were made out of very fine wire netting. The wasps were provided with a 10% honey solution and flowering *Sinapis* plants. They were in a room where the temperature was kept constant at 20ºC, 70-80% RH and a 16L:8D photoperiod.

Newly emerged flea beetle larvae were transferred to radish plants of about 15 cm height. Each time as many larvae as possible were transferred. After at least 4 days the radish plant with the larvae was put inside on of the cages. There were 3 cages, one for species A, one for species B, and one for species C. After 24 hours the radish plants were taken out of the cages. When the larvae in the radish leaves were final instars, the leaves they were in were picked and put into vials with a layer of peat and vermiculite. The larvae left the leaves and dug into the peat/vermiculite layer when they were ready to pupate. It was checked if some larvae developed into wasps. The wasps which emerged were put back into their own species' cage.

# 3. Results and discussion

## 3.1. Testing the seasonal variability in defences of *Barbarea*

Table 4 shows the results of the bioassays with the adult flea beetles collected on *Barbarea* in the field. No bioassays were done with offspring of the non-feeder collected in Kværkeby 3 on 20-04-06. Therefore, it is not 100% sure if this non-feeder is also really a susceptible flea beetle. However, the two non-feeding males collected in Amager were crossed with a susceptible female from the isogenic line in the laboratory (de Jong and Nielsen 2002). All larvae tested from these crossings (28 for the one collected 09-05-20, 30 for the one collected 26-5-06) turned out to be non-feeders as well. This finding shows that the two non-feeders collected in Amager really are susceptible to the defence mechanisms of *Barbarea*.

Table 4. The results of the bioassays with the adult flea beetles collected on *Barbarea*.

| *Starting date* | *Location* | *Nr of beetles* | *Percentage females* | *Percentage non-feeders* |
|---|---|---|---|---|
| 20-04-06 | Kværkeby 3 | 24 | 16.67 | 4.17 |
| 26-04-06 | Ejby | 24 | 41.67 | 0 |
| 02-05-06 | Kværkeby 3 | 30 | 33.33 | 0 |
| 02-05-06 | Kværkeby 1 | 26 | 53.85 | 0 |
| 03-05-06 | Ejby | 30 | 46.67 | 0 |
| 04-05-06 | Amager | 1 | 100 | 0 |
| 09-05-06 | Amager | 1 | 0 | 100 → susceptible! |
| 26-05-06 | Amager | 1 | 0 | 100 → susceptible! |
| 31-05-06 | Amager | 1 | 0 | 0 |

What is also quite striking about the results in table 4 is the fact that the beetles collected in Kværkeby 3 were mostly males. The first collection date only 4 out of 24 beetles were female. The second collection date 10 out of 30 beetles collected were female.

## 3.2. Testing for enemy-free space

### *3.2.1. Plant characteristics*

Figure 3 shows the development of the frequency of plants with flowering stalks in all locations combined. Figure 4 shows the development of the frequency of flowering plants in all locations combined. Figure 5 shows the development of the biomass of the plants in all locations combined. The error bars show the range. Some calculations had to be done before I was able to make the graphs. The following method applies to the flowering stalk data, the flowering data as well as the biomass data. As said, plant data in all locations but one (Kværkeby *Sinapis*) was measured in 5 plots per plant species. The average value of each of the 5 plots was calculated. Per location the average of these values per plot was taken. The result of these calculations was an average of frequency of plants with flowering stalks, frequency of flowering plants, and biomass per plant for each separate location per collection date.

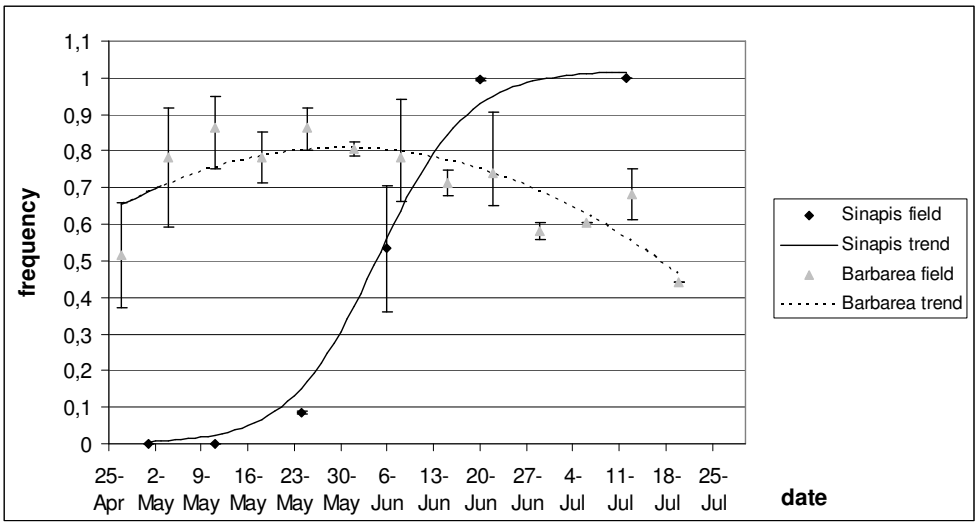


Figure 3. Frequencies of plants with flowering stalks. This graph shows the data of all locations combined. Error bars represent range. Trend lines were calculated using non-linear regression.

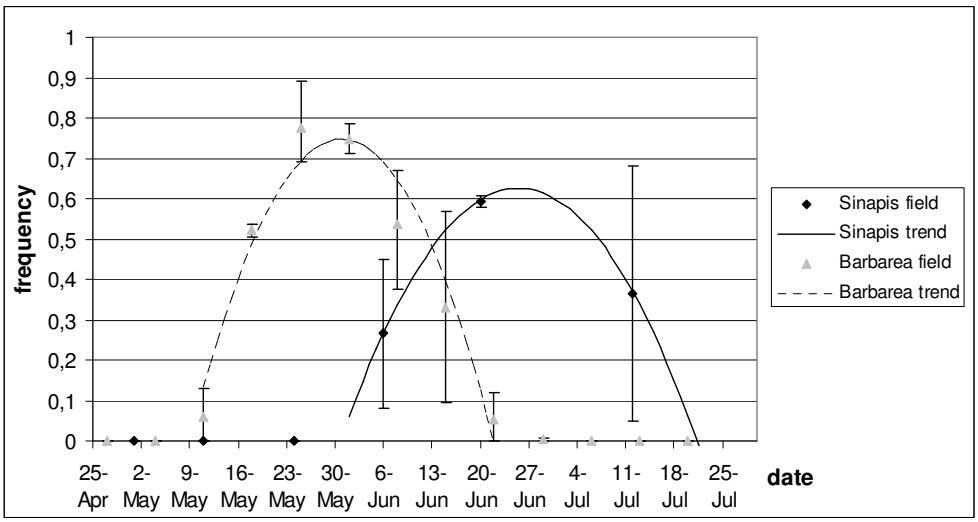


Figure 4. Frequencies of flowering plants. This graph shows the data of all locations combined. Error bars represent range. Trend lines were calculated using non-linear regression.

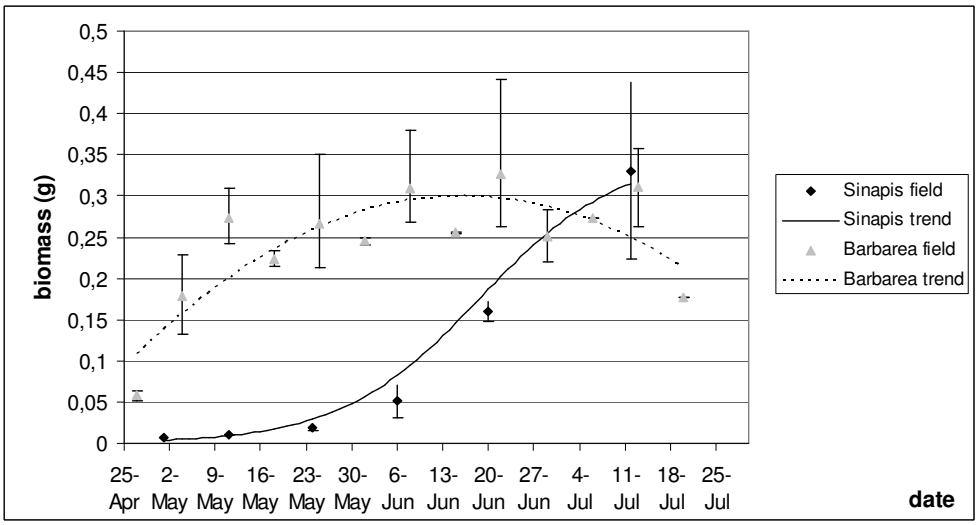


Figure 5. Average biomass per plant. This graph shows the data of all locations combined. Error bars represent range. Trend lines were calculated using non-linear regression.

The *Sinapis* data collected in Kværkeby and Suserup were always collected the same day. Therefore, each collection date the average of the values of these two locations was calculated. The results are shown in figure 3, 4 and 5 as the *Sinapis* series.

The *Barbarea* data collected in Kværkeby 1 and Kværkeby 3 were always collected the same day. So for these dates the average of the two locations was taken, resulting in one value for Kværkeby per collection date. However, the *Barbarea* data of Kværkeby, Ejby and Amager were collected on different dates. To nevertheless give an idea of the development of *Barbarea* in all locations combined, the collection period (25 April until 17 July) was divided into weeks. For each of these weeks, the average value of the data collected in the study areas in this week was calculated. The resulting value, the average of the *Barbarea* data of all locations in that week, was allocated to the $4^{th}$ day of the week. These values are shown in figure 3, 4 and 5 as the *Barbarea* series.

In all graphs, for both the *Barbarea* and the *Sinapis* series, regression lines were calculated using SPSS software. In 4 cases a parabolic curve seemed to produce the best fitting line, in 2 cases a logistic growth curve seemed to be the best option. In the data series concerning flowering frequencies the points with value 0 were excluded to produce the regression lines. All trend lines were significant ($P<0.05$), except the one for flowering frequencies of *Sinapis*. No F-value could be calculated for this trend line, because only 3 points were used to calculate the line.

Table 5 shows the characteristics of all the trend lines calculated. All parabolic lines are calculated by the following formula: $y = b_0 + b_1 * t + b_2 * t^2$. The logistic lines are calculated by this formula: $y = 1 / (1 / u + (b_0 * (b_1^t)))$, where u is the upper limit of the graph. In both cases t stands for time in days. For the *Barbarea* series t=1 is on 27 April 2006, for the Sinapis series t=1 is on 1 May 2006.

In table 5 it is also shown when the regression lines peak. For the parabolic lines it is easy to see and calculate where the peak is. However, to determine a peak for the logistic lines is less straightforward. For the logistic lines I chose to determine the time to the peak as twice the time to where the curve changes from increasing growth to decreasing growth.

Table 5. Characteristics of the regression lines shown in the figures about plant phenology.

| | Flowering | | Flowering stalk | | Biomass | |
|---|---|---|---|---|---|---|
| plant | *Barbarea* | *Sinapis* | *Barbarea* | *Sinapis* | *Barbarea* | *Sinapis* |
| regression form | parabolic | parabolic | parabolic | logistic | parabolic | logistic |
| u | - | - | - | 1.02 | - | 0.35 |
| $b_0$ | -1.14E+00 | -2.38E+00 | 6.43E-01 | 2.08E+02 | 9.97E-02 | 2.97E+02 |
| $b_1$ | 1.08E-01 | 1.06E-01 | 9.50E-03 | 8.60E-01 | 7.82E-03 | 9.11E-01 |
| $b_2$ | -1.54E-03 | -9.41E-04 | -1.37E-04 | - | -7.64E-05 | - |
| adjusted R square | 0.91 | 1.00 | 0.58 | 0.91 | 0.62 | 0.94 |
| F-value | 30.86 | - | 9.37 | 51.28 | 10.59 | 86.68 |
| P-value | 0.0037 | - | 0.0051 | 0.002 | 0.0034 | 0.0007 |
| peak | 1-jun | 27-jun | 1-jun | 12-jul | 16-jun | 9-aug |

What can be seen in all three graphs is that on average *Barbarea* grows and develops earlier in the season than *Sinapis*. This conclusion can also be drawn from the peak dates calculated and shown in table 5. In all cases it is clear that *Barbarea* peaks at an earlier date than *Sinapis*. Because plant biomass is the most influential for the flea beetles, graph 5 is the most important graph concerning plant characteristics. Also this graphs shows that at the beginning of the season *Barbarea* plants have already much more leaf biomass than *Sinapis* plants. Also graph 3 shows that on average *Barbarea* plants develop a flowering stalk earlier in the season than *Sinapis* plants, with the result that they are also able to flower earlier as seen in figure 4.

It was obvious in the field that after the *Barbarea* plants had flowered, the leaves started to wilt. All plants collected in the field to measure biomass were not yet flowering or flowering, and this might have had the effect that after the flowering peak as seen in figure 4 the estimates of biomass were too high. To show that indeed available leaf biomass decreased after flowering, on 20 June again 5 plants of the 4 largest height-diameter class combinations were collected in the field, only this time it was made sure the plants had already flowered. Leaf biomass of these plants was determined. The results are shown in figure 6. It is obvious that indeed after flowering on average the biomass of plants of a class is smaller than the biomass of the same class before or during flowering. However, this effect is not taken into account in figure 5. So it is very likely that in reality the biomass series as shown in this figure would decrease after the flowering peak as shown in figure 4. Knowing this, it has no real influence on the conclusions that can be drawn from figure 5 concerning the date at which flea beetle larvae can use the two different plant species. Still it is clear that *Barbarea* plants can be used earlier in the season than *Sinapis* plants.

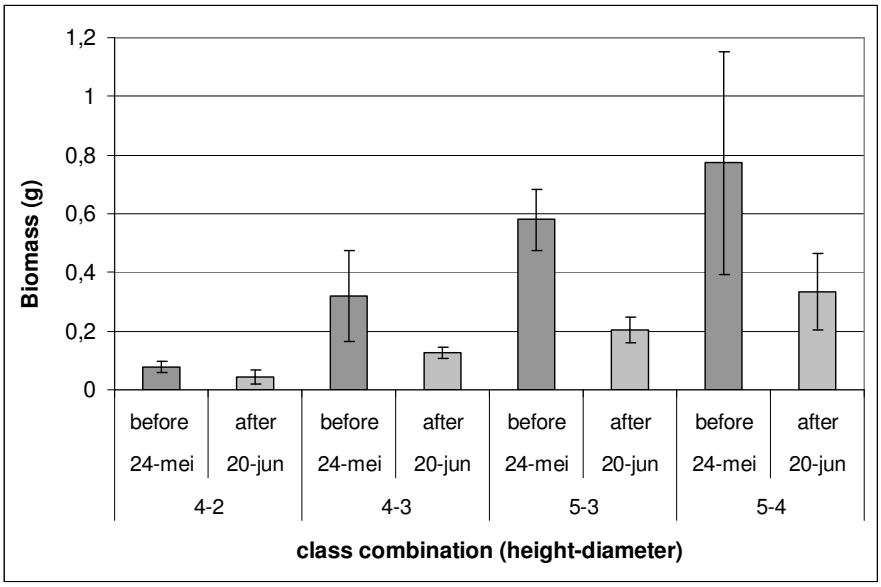


Figure 6. Average biomass (g) of the largest height-diameter classes of *Barbarea* still present in the field late in the season. Biomass was measured before or during flowering (the "before" series) and after flowering (the "after" series). Error bars represent standard deviation.

### *3.2.2. Parasitism rates in Ejby*

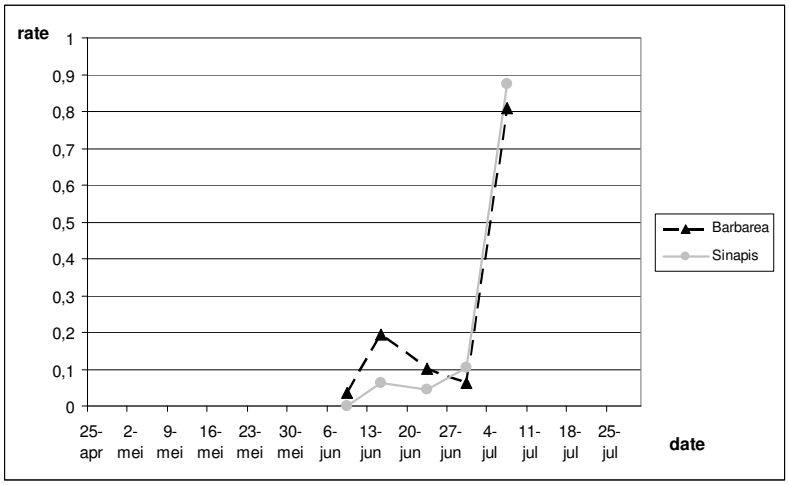


Figure 7. Overall parasitism rates of 3rd instar flea beetle larvae on *Barbarea* and *Sinapis* in Ejby.

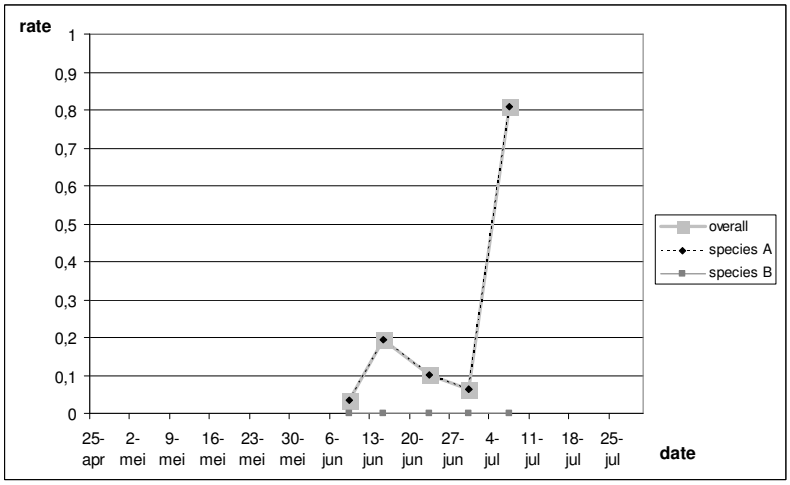


Figure 8. The parasitism rate of 3rd instar flea beetle larvae on *Barbarea* in Ejby. The three lines show the parasitism rate of both wasp species together, the rate of species A and the rate of species B.

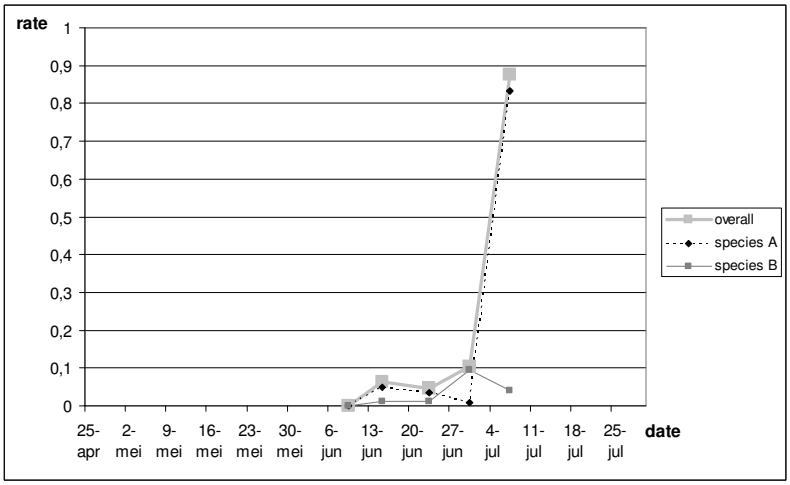


Figure 9. The parasitism rate of 3rd instar flea beetle larvae on *Sinapis* in Ejby. The three lines show the parasitism rate of both wasp species together, the rate of species A and the rate of species B.

Figure 7 shows the development of the overall parasitism rate of flea beetle larvae collected on both *Sinapis* and *Barbarea* in Ejby. Overall parasitism rates means that it was not taken into account which parasitoid species was involved. Figure 8 shows the parasitism rate of flea beetle larvae collected on *Barbarea* in Ejby per parasitoid species. Figure 9 shows the same, but then for larvae collected on *Sinapis*.

Figure 7 shows that on both plant species 2 main peaks of parasitism are visible. The first peak can be seen on 15 June 2006, the second on 7 July 2006. So there are approximately 3 weeks between the two peaks. As figures 8 and 9 show, the peaks are caused by parasitism of species A. Species B, which was only found on *Sinapis* and not on *Barbarea*, peaks only once on 30 June 2006.

The second peak of species A is clearly much higher than the first peak. The peaks can be explained by considering the first peak the first generation of wasp A, and the second peak the second generation of wasp A. In the lab the pupation of wasp A took approximately 12 days. It is often seen that it takes roughly 24 hours after the pupation before a parasitoid wasp is able to oviposit (Liu 2001). Furthermore only third instar flea beetle larvae were collected in the field. It takes a newly emerged larva around 6 days to become a third instar. Taking this all into account, 3 weeks between two generations collected in third instar larvae seems not unlikely.

Opposite of what was found by De Jong and Nielsen (unpublished data), this year in Ejby parasitism on *Barbarea* was higher than on *Sinapis* early in the season. The difference was largest on 15 June 2006. This collection date parasition on *Barbarea* was 19.5%, while it was only 6.2% on *Sinapis*. This makes parasitism on *Barbarea* significantly higher than on *Sinapis* for this date (Chi-square = 31.21, df=1, P<0.001). On 23 June 2006 parasitism on *Barbarea* was 10.2%, while on *Sinapis* it was only 4.6%. On this date parasitism was also significantly higher on *Barbarea* than on *Sinapis* (Chi-square = 5.04, df=1, P<0.025). On the other dates no difference in parasitism between the two plant species was found.

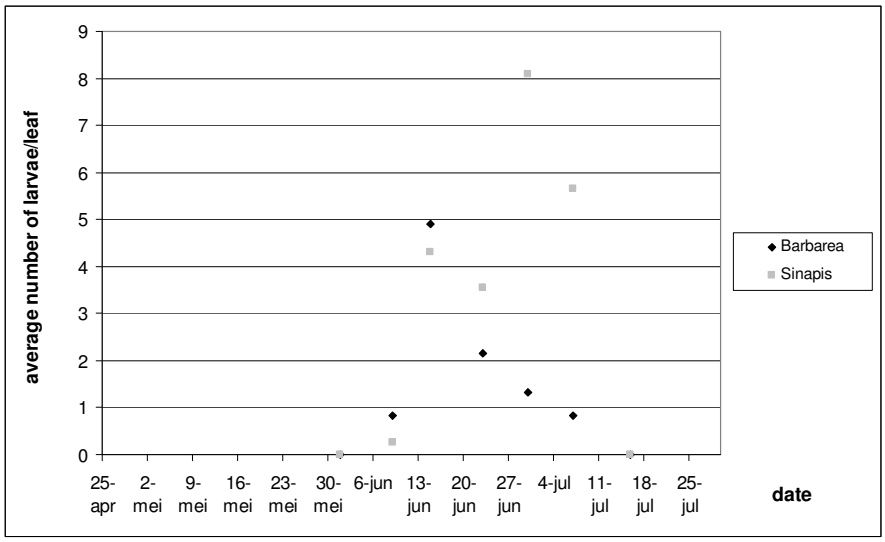


Figure 10. The average number of 3rd instar flea beetle larvae per leaf in Ejby.

Figure 10 shows the density of 3rd instar larvae in the leaves collected in Ejby. The figure shows that the whole season of flea beetle larvae is included in the research. Before the first larvae were collected, approximately one week earlier it was already tried to collect larvae in the field. Leaves were collected, but all the larvae in these leaves were 1st or 2nd instar. The first time it was really possible to collect 3rd instar larvae was on 9 June 2006. The last date it was possible to collect larvae in the field was 7 July 2006. One week later, on 16 July 2006, it

was tried to collect larvae, but it was impossible because numbers were too low, both on *Barbarea* and *Sinapis*.

Figure 10 indicates that flea beetle larvae peak earlier on *Barbarea* than on *Sinapis*. The highest densities of flea beetle larvae on Barbarea were found 15 June 2006, while the highest densities of larvae on Sinapis were found 30 June 2006. This means that this season in Ejby larvae on *Barbarea* peaked approximately 2 weeks earlier than the larvae on *Sinapis*.

Just before the collection on 23 June 2006 the *Sinapis* plot in Ejby was mown. Fortunately enough most plants were only "decapitated", leaving most leaves intact. Also the area was quite hard to mow, because of the slope and some difficult corners, so there were still some completely intact plants left. Collections on Sinapis in Ejby on 23 June and 30 June were not really influenced by the mowing: it was still quite easy to collect high numbers of leaves and larvae. On these dates even the decapitated plants were still alive and served as food for the flea beetle larvae. However, on 7 July the mowing might have had an influence on the collections. A large part of the decapitated plants started to suffer from the mowing, and had died so they could not longer serve as food for the flea beetle larvae. Therefore larvae were collected only on the undamaged plants. This might have had the result that larvae were clustered on the undamaged plants, and that the estimate of density I made was therefore too high for that collection date. However, I believe that the effect of the mowing was not really important. This is because one collection date earlier the mowing still did not have a lot of influence on the plants, and only 3$^{rd}$ instar larvae were used to calculate the densities. This means that these larvae already had most probably been at least six days on the plant they were collected on. The eggs they emerged from were laid even a few days earlier. Therefore the density of 3$^{rd}$ instar larvae in the leaves in fact reflects the situation of more than a week earlier. Because a week earlier the situation was still quite normal, and because the flea beetle larvae are not very mobile, I believe that also the data collected the last collection date is still reliable.

It is interesting to see how much seasonal overlap the flea beetle population and the populations of wasps have. The following formula can be used to calculate seasonal overlap of a population of parasitoids and its host larvae (Feder 1995):

$$overlap = \sum_{i=0}^{k}(p_i \times f_i) / \sqrt{(\sum_{i=0}^{k} p_i^2 \times \sum_{i=0}^{k} f_i^2)}\,, \qquad (1)$$

where $p_i$ represents the proportion of the adult parasitoid population in period i, and $f_i$ represents the proportion of fly larvae present at this time. In this case $p_i$ is calculated by dividing the parasitism rate at moment i by the sum of all parasitism rates. To calculate $f_i$ the density of flea beetle larvae at moment i was divided by the sum of all densities.

Feder (1995) did not use parasitism rate to calculate $p_i$. During his research he counted actual numbers of adult parasitoids in the neighbourhood of the host larvae. Because parasitism and density might be related, the method of Feder (1995) is better than the method used here. However, no data about numbers of adult parasitoids are present, so the only way to determine seasonal overlap is to use parasitism rates.

Seasonal overlap calculated for wasp A and flea beetle larvae on *Sinapis* was 54.0%. Seasonal overlap for wasp A and flea beetle larvae on *Barbarea* was 41.3%. Figure 11 and 12 show the relation between the development of the population of wasp A and the development of the population of flea beetle larvae on respectively *Sinapis* and *Barbarea*. What is most striking from the figures is that it seems that the lower seasonal overlap on *Barbarea* than on *Sinapis* is mostly caused by the last collection date. Figure 12 shows that before this collection date the development of wasp A and the flea beetle larvae on *Barbarea* are relatively similar, even more similar than the development of wasp A and larvae on *Sinapis*. However, because of the high parasitism rate and the low density of larvae on *Barbarea* on the last collection date, the seasonal overlap of the flea beetle larvae population and the population of wasp A in Ejby is lower on *Barbarea* than on *Sinapis*.

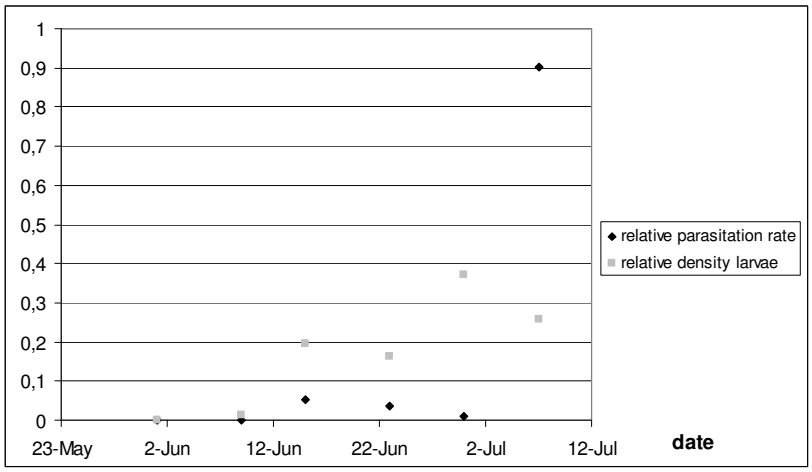


Figure 11. The development of the relative parasitism rate ($p_i$) of wasp A and the relative density of flea beetle larvae ($f_i$) on *Sinapis* in Ejby.

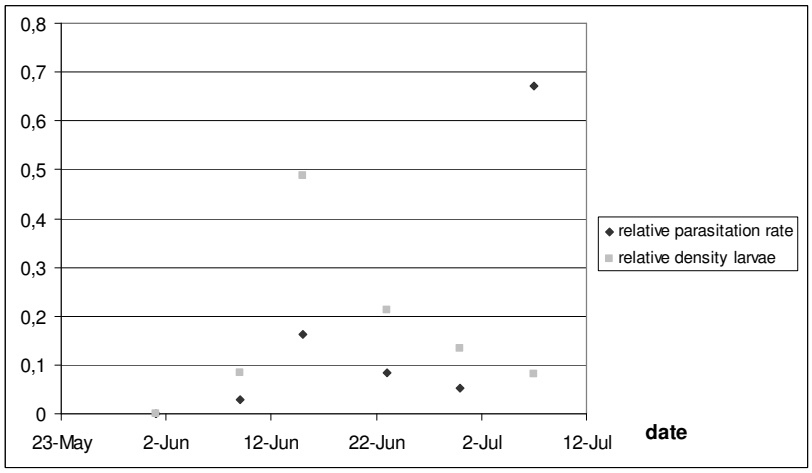


Figure 12. The development of the relative parasitism rate ($p_i$) of wasp A and the relative density of flea beetle larvae ($f_i$) on *Barbarea* in Ejby.

Seasonal overlap calculated for the population of wasp B and flea beetle larvae on *Sinapis* in Ejby was 92.4%. No seasonal overlap could be calculated for wasp B and larvae on *Barbarea*, because parasitism of wasp B was always 0 on this plant species. Why the seasonal overlap for wasp B on Sinapis is so high can be seen in figure 13. The development of the two populations is very similar: when there is an increase or decrease in the density of larvae, there is also respectively an increase or decrease in parasitism rate.

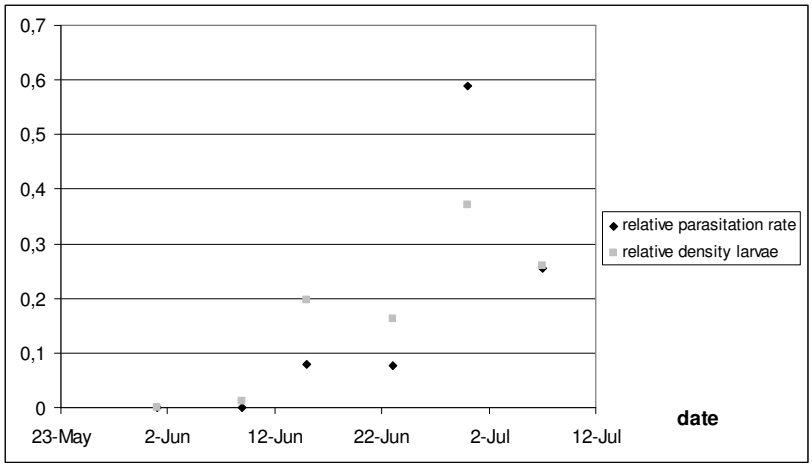


Figure 13. The development of the relative parasitism rate ($p_i$) of wasp B and the relative density of flea beetle larvae ($f_i$) on *Sinapis* in Ejby.

### *3.2.3. Parasitism rates in Kværkeby, Amager and Suserup*

Figure 14 and 15 show the parasitism rate of respectively wasp A and wasp B in Amager Ørestad, Kværkeby and Suserup. These three areas were the ones apart from Ejby where data about parasitism rates were collected more than once.

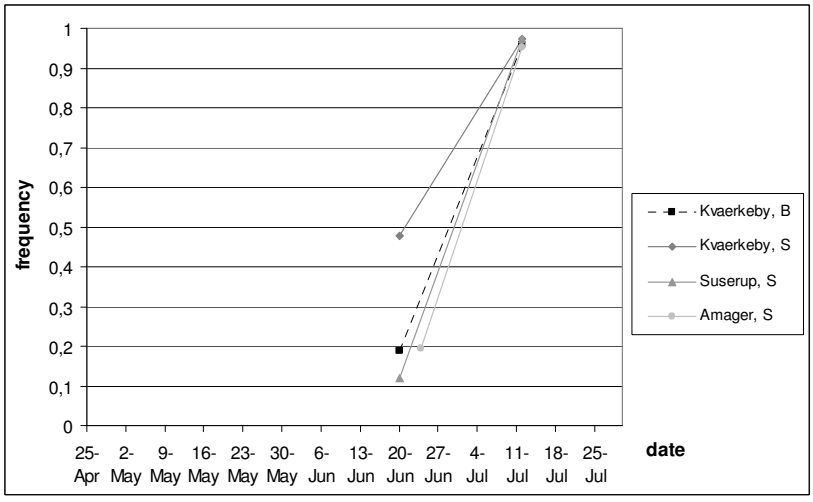


Figure 14. Parasitism rates of wasp A in Kværkeby, Suserup and Amager. In each area 2 samples were taken, an early one and a late one. In all areas larvae were collected on *Sinapis* (S), only in Kværkeby larvae were also collected on *Barbarea* (B).

Figure 14 shows that in all areas parasitism by wasp A was much higher in the late collection (12 July) than in the early collection (20 June for Kværkeby and Suserup, 24 June for Amager Ørestad) (Kværkeby *Barbarea*: Chi-square=246.52, df=1, P<0.001; Kværkeby *Sinapis*: Chi-square = 49.29, df=1, P<0.001; Suserup *Sinapis*: Chi-square = 153.30, df=1, P<0.001; Amager *Sinapis*: Chi-square = 44.39, df=1, P<0.001). This corresponds with what was also seen in Ejby.

Parasitism on *Barbarea* in Kværkeby is much lower than parasitism on *Sinapis* in the same area early in the season (Chi-square = 29.20, df=1, P<0.001). Parasitism by wasp A late in the season was extremely high in all areas on both plants. In all samples collected parasitism was around 96%. In Kværkeby late in the season there was no difference in

parasitism between larvae on *Barbarea* and larvae on *Sinapis* (Chi-square = 0.36 df=1, P>0.05).

Parasitism rates for wasp A were also different between the 3 locations. Parasition on *Sinapis* early in the season in Kværkeby was significantly higher than parasitism on *Sinapis* early in the season in Suserup (Chi-square = 58.52, df=1, P<0.001) and in Amager (Chi-square = 16.63, df=1, P<0.001). Parasitism early in the season on *Sinapis* was not different between Suserup and Amager (Chi-square = 3.43, df=1, P<0.10).

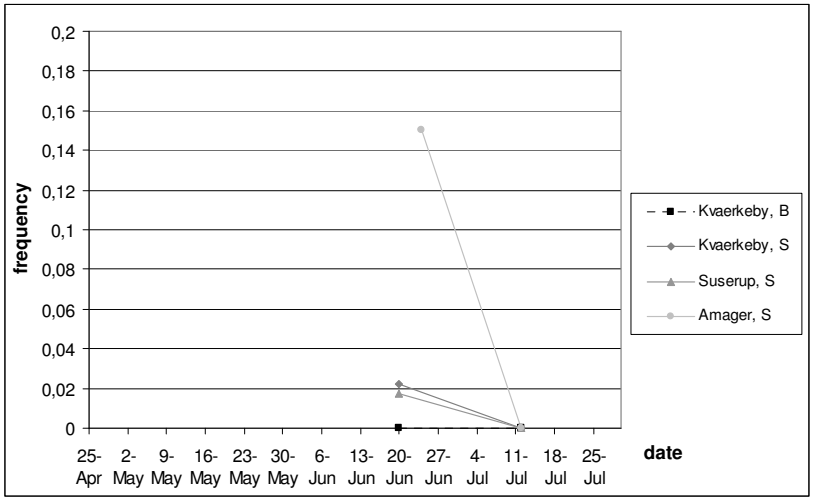


Figure 15. Parasitism rates of wasp B in Kværkeby, Suserup and Amager. In each area 2 samples were taken, an early one and a late one. In all areas larvae were collected on *Sinapis* (S), only in Kværkeby larvae were also colleted on *Barbarea* (B).

Figure 15 shows that, similar to what was found in Ejby, flea beetle larvae collected on *Barbarea* in Kværkeby were never parasitized by wasp species B. On all locations flea beetle larvae collected early in the season on *Sinapis* were parasitized by this wasp. However, parasitism of wasp B on *Sinapis* in Kværkeby and Suserup was very low, both approximately 2%. The highest parasitism by far was found in Amager Ørestad on *Sinapis*: 15%. Parasitism by wasp B in Amager early in the season was significantly higher than parasitism of this wasp early in the season in Kværkeby (Chi-square = 9.44, df=1, P<0.01) and in Suserup (Chi-square = 29.59, df=1, P<0.001). Late in the season parasitism by wasp B was absent in all locations on both plants species.

### *3.2.4. Experimental plots*

Due to the fact that the seedlings in the plots did not grow as well as we expected them to grow, it was impossible to collect larvae early in the season in the plots. However, late in the season it was possible to collect some larvae in the plots, and to determine the parasitism rate of these larvae. On 12 July 2006 in total 32 larvae were collected in the *Barbarea* plots, and 59 in the *Sinapis* plots. Parasitism on *Barbarea* was 100%, on Sinapis 96,8%. The difference between the parasitism on *Barbarea* and *Sinapis* in the plots was not significant (Chi-square = 0.72, df=1, P>0.05).

*3.2.5. Plant data, density data and parasitism data combined*

Figure 10 shows that flea beetle larvae peak earlier on *Barbarea* than on *Sinapis* in Ejby. The height of the peak in the figure is not so relevant, because *Sinapis* leaves are on average larger than *Barbarea* leaves, so they can contain more larvae. The question that arises now is: can the plant characteristics collected show us why flea beetle larvae peak earlier on *Barbarea* than on *Sinapis*. The answer to this question seems to be "yes". As seen before, leaf biomass, and thus food for flea beetle adults and larvae, is on average available earlier on *Barbarea* than on *Sinapis*. This means that flea beetle adults are able to start to eat more, earlier in the season, so they are ready earlier to lay eggs. The same amount of food is available several weeks later on *Sinapis*, with the result that the population on *Sinapis* is only able to start to build up some weeks later.

When you look at figure 7 it seems like, looking at the whole season, parasitism in Ejby this year was higher for larvae on *Barbarea* than for larvae on *Sinapis*. Also, if one would calculate the parasitism of the whole season by simply dividing the total number of wasps which emerged from all larvae collected by the total number of wasps and beetles which emerged from the larvae, the result would be that parasitism rate on *Barbarea* was higher than on *Sinapis* (16.2% for *Barbarea* and 12.6% for *Sinapis*). However, this method is not correct for calculating the effect of parasitism on a population for a whole season. Firstly, this is caused by the fact that not every collection date exactly the same number of larvae was collected. The more larvae are collected on one date, the higher the influence of the parasitism rate of that collection date on the outcome of the parasitism rate of the whole season. The outcome of the parasitism rate of the whole season should be independent of the number of larvae collected per date. Secondly, if the parasitism rate of the whole season is calculated like this, it does not take into account the number of larvae present at the time of the measured parasitism rates. The effect of the parasitism rate at one moment in time on the overall parasitism rate of the whole season is determined by what proportion of the whole seasonal population (of, in this case, larvae) is present at the time the parasitism rate is measured. Therefore, to give a good estimate of the parasitism rate of the whole season also data about population size at the collection dates is needed.

Because it is quite impossible to get an impression on absolute number of larvae in the field, I thought of a method to get an impression on relative number of larvae in the field. Therefore I used an indication for density and an indication for biomass. The indication for density I used is the number of third instar larvae per leaf collected in the field (LpL). The indication for biomass is the average biomass of the plants in the field (B). B is obtained making use of the regression lines calculated for the average biomass per plant. I have the impression that the total number of plants does not change that much during the larval season. Therefore total biomass in the field is mainly influenced by the average biomass of the plants.

For each collection date the density has to be multiplied by the biomass, to get an indication for total number of larvae in the field at each collection date ($NLD_i$). So for each collection date the following formula has to be used:

$$NLD_i = Lpl_i \cdot B_i \qquad (2)$$

The sum of all the results of formula 2 has to be taken. The result will be called the sum of the NLD (SNLD):

$$SNLD = \sum_{i=0}^{k} NLD_i = \sum_{i=0}^{k} Lpl_i \cdot B_i \tag{3}$$

Now for each collection date the result of formula 2 has to be divided by the result of formula 3, to get the relative $NLD_i$ ($RNLD_i$) for each collection date:

$$RNLD_i = \frac{NLD_i}{SNLD} = \frac{Lpl_i \cdot B_i}{\sum_{i=0}^{k} Lpl_i \cdot B_i} \tag{4}$$

Parasitism rates for each collection date ($PRD_i$) are known. For each collection date the $PRD_i$ has to be multiplied with the $RNLD_i$, to get the relative parasitism rate ($RPR_i$):

$$RPR_i = PRD_i \cdot RNLD_i = PRD_i \cdot \frac{LpL_i \cdot B_i}{\sum_{i=0}^{k} LpL_i \cdot B_i} = \frac{PRD_i \cdot LpL_i \cdot B_i}{\sum_{i=0}^{k} LpL_i \cdot B_i} \tag{5}$$

To finally come up with the parasitism rate of the whole season (SPR) the sum of all the RPRs has to be taken:

$$SPR = \sum_{i=0}^{k} RPR_i = \frac{\sum_{i=0}^{k} PRD_i \cdot LpL_i \cdot B_i}{\sum_{i=0}^{k} LpL_i \cdot B_i} \tag{6}$$

The assumptions made for this model:
- total number of plants stays the same during the larval season;
- the biomass $B_i$ is proportionally related to the total number of leaves. B does not have to be equal to the number of leaves, because the $RNLD_i$ is calculated. The $RNLD_i$ only shows when the population larvae in the field is largest, and therefore gives a weight to the $PRD_i$. It does not say anything about absolute number of larvae in the field, what also is not necessary to calculate the SPR.

Two different estimates were made, one taking into account only the density of larvae, the other one also taking into account the average biomass of the plants. The first estimate thus excludes $B_i$ from the formulas 2-6 (so for example $NLD_i$ then equals $Lpl_i$), the second estimate was calculated with formulas 2-6 exactly as described above.

The result of the estimate made without the biomass data is that the overall parasitism rate of the whole season on *Barbarea* in Ejby is 19.5%, while on *Sinapis* it is 28.5%. The difference between the two plant species (9.0%) is caused by a 5.1% higher parasitism of wasp B on *Sinapis* and a 3.9% higher parasitism of wasp A on *Sinapis*.

The result of the estimate made with the biomass data is that the overall parasitism rate of the whole season on *Barbarea* in Ejby is 19.1%, while on *Sinapis* it is 34.1%. The difference between the two plant species (15.0%) is caused by a 5.5% higher parasitism of wasp B on *Sinapis* and a 9.5% higher parasitism of wasp A on *Sinapis*.

Figure 16 shows the development of the relative parasitism rate calculated without taking biomass into account. It becomes evident from this graph, if you compare it to graph 7, that for the flea beetle population on *Barbarea* the first peak in the parasitism rate is more important for the overall seasonal parasition than the second one. For the flea beetle population on *Sinapis* the second peak in parasitism rate has most influence on the total parasitism rate of the whole season. Figure 17, which shows the cumulative relative parasitism rate, shows that only the last collection date determined that overall parasitism rate was higher on *Sinapis* than on *Barbarea*.

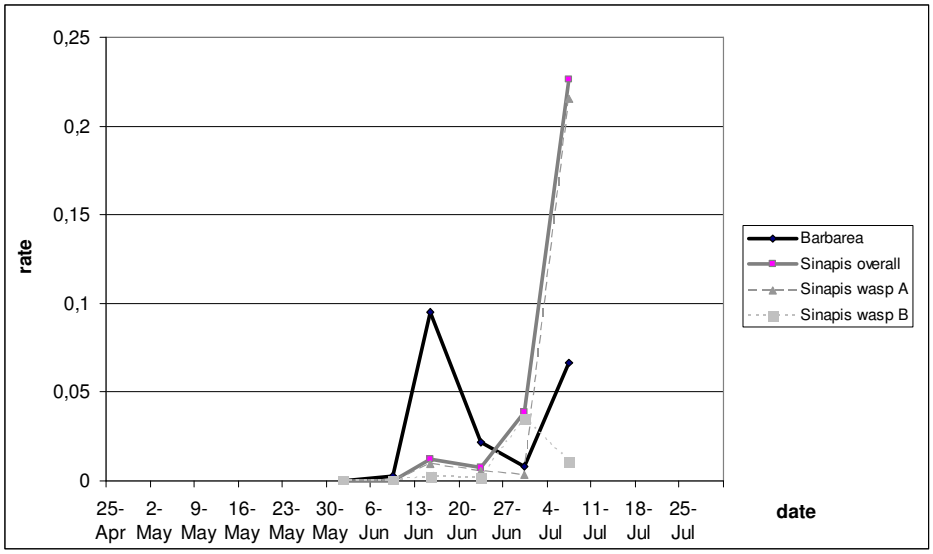


Figure 16. The development of the relative parasitism rate ($RPR_i$) of flea beetle larvae in Ejby, calculated without taking the biomass into account.

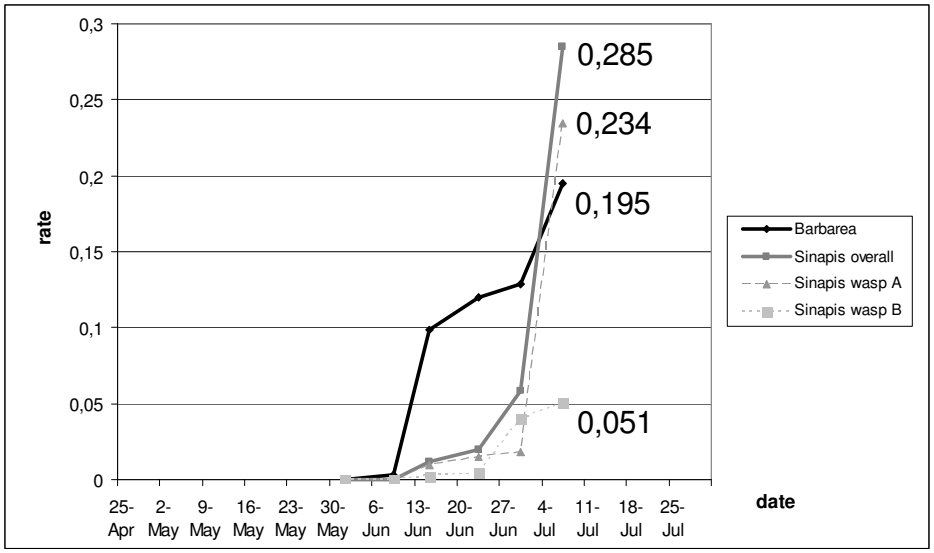


Figure 17. The development of the cumulative relative parasitism rate of flea beetle larvae in Ejby, calculated without taking the biomass into account. The values shown are the final estimates of the parasitism rate of the whole season (SPR).

Figure 18 and 19 show the same as figure 16 and 17, but now the calculations were done with the biomass data incorporated. These graphs show the same developments, only this time the difference of between parasitism rates of wasp A on *Sinapis* and *Barbarea* is bigger.

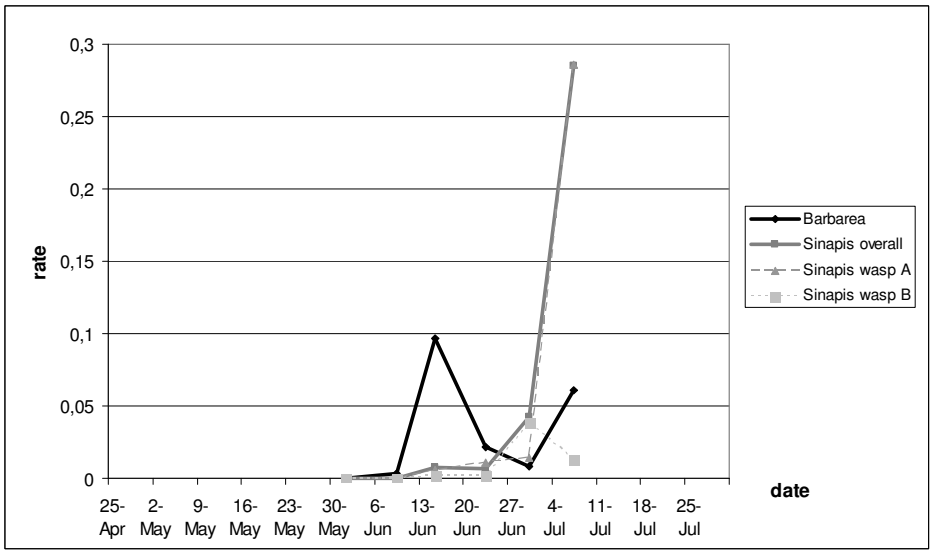


Figure 18. The development of the relative parasitism rate ($RPR_i$) of flea beetle larvae in Ejby, calculated with taking the biomass into account.

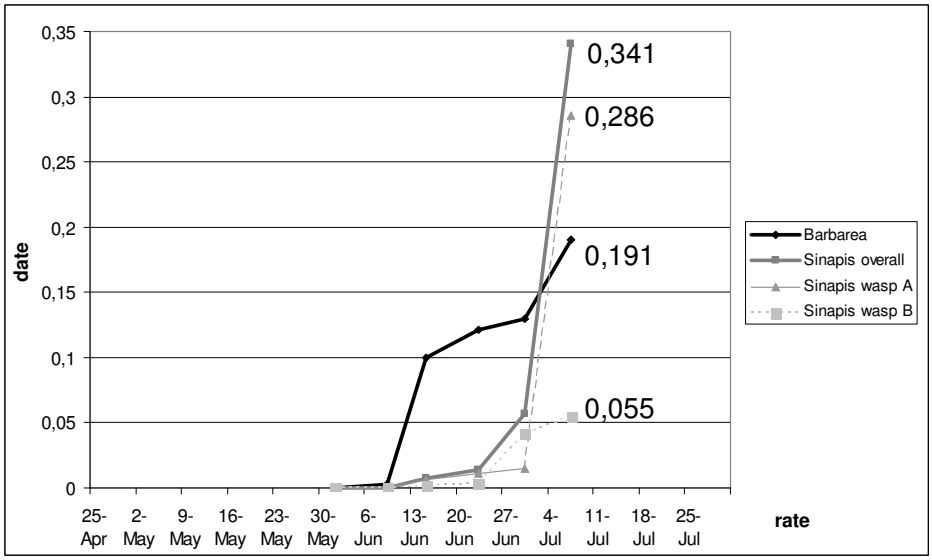


Figure 19. The development of the cumulative relative parasitism rate of flea beetle larvae in Ejby, calculated without taking the biomass into account. The values shown are the final estimates of the parasitism rate of the whole season (SPR).

## 3.3. Testing for the importance of *A. brevicauda* as natural enemy

Wasp species A turned out to be *Diospilus morosus* (Hymenoptera: Braconidae). Wasp species B turned out to be *Aneuclis brevicauda* (Hymenoptera: Ichneumonidae). This was surprising, because initially it was thought that *A. brevicauda* was the most important natural enemy of flea beetles (de Jong and Nielsen unpublished data). However, parasitism by *D. morosus* seems to be more common than parasitism by *A. brevicauda*. Figure 20 shows pictures of males and females of both wasp species.

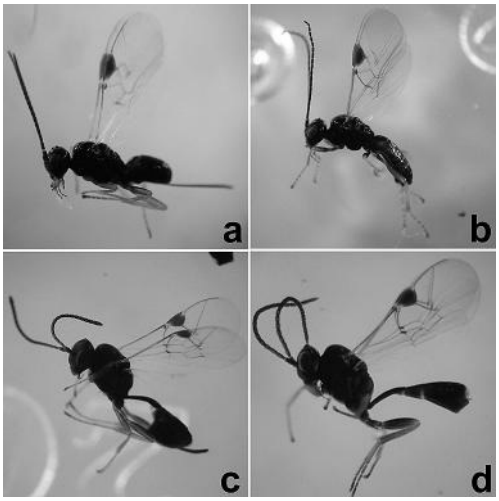


Figure 20. Enlarged pictures of a) a female *Diospilus morosus*, b) a male *Diospilus morosus*, c) a female *Aneuclis brevicauda* and d) a male *Aneuclis brevicauda*.

# 4. Conclusions and general discussion

## 4.1. Answers to research questions

At the start of the research I formulated several questions that had to be answered to get to the answer to the main question: Does *Barbarea* provide an enemy-free space to *Phyllotreta nemorum*? In this chapter these questions are answered, and conclusions are drawn making use of these answers.

The first question that is addressed is: Does *Barbarea* grow and develop earlier in the season than *Sinapis*? The answer to this question is: yes. In this research it is found that in East Denmark food biomass is available earlier in the season on *Barbarea* than on *Sinapis*. It is also found that *Barbarea* plants on average flower earlier in the season than *Sinapis* plants. All in all it is clear that flea beetle larvae do have the possibility to use *Barbarea* earlier than *Sinapis*. A possible explanation for this finding is that *Barbarea* is able to overwinter as a rosette (Macdonald and Cavers 1991), while *Sinapis* is not (Warwick et al. 2000).

Then the next question that arises is: Do larvae of *P. nemorum* really start to use *Barbarea* as a host plant earlier in the season than *Sinapis*? Also this question can be answered positively. It was found that the population of flea beetle larvae peak several weeks earlier on *Barbarea* than on *Sinapis*. By the time parasitism rates are extremely high at the end of the season, the population density of larvae on *Barbarea* is very low, while on *Sinapis* it is still relatively high. Natural enemies can restrict reproduction of herbivores to periods of low activity of these natural enemies, however only few examples are found where this really happens (Hopkins and Memmott 2003). Also in this study this phenomenon is not observed. Maybe this is because parasitism pressure on the population of flea beetle larvae on *Sinapis* is not high enough to restrict the flea beetles to an earlier reproduction (on a different host).

So *Barbarea* can be, and in fact is, used earlier by flea beetles than *Sinapis*. Now the question is: Are parasitism levels of *P. nemorum* larvae on *Barbarea* lower than those on the *Sinapis*, and if yes, when? Because it was found that there are 2 parasitoid species, there are 2 different answers to this question. If you look at *Aneuclis brevicauda*, the least abundant of the two species, then the answer is: yes, there are several moments at which parasitism of *A. brevicauda* was higher for larvae feeding on *Sinapis* than for larvae feeding on *Barbarea*. This observation is seen in both Ejby and Kværkeby. In fact, never a single *A. brevicauda* adult emerged from larvae collected on *Barbarea*. Why and how this could have happened is explained later in this chapter.

If you look at *Diospilus morosus*, the more abundant of the two species, the answer to this question is not straightforward. The situation in Ejby is different from the situation in Kværkeby. In Ejby it was found that, in contrast to what was found earlier by de Jong and Nielsen (unpublished data), on most collection dates the parasitism rate was similar on *Barbarea* and *Sinapis*, except on two dates where parasitism was higher on *Barbarea* than on *Sinapis*. The biggest difference was found early in the season, in the second week that flea beetle larvae were present.

At the end of the season parasitism in Ejby was very high on both *Barbarea* and *Sinapis*. This finding indicates that *D. morosus* is very well capable of locating and parasitizing flea beetle larvae feeding on *Barbarea* plants, and that the flea beetle larvae on *Barbarea* do not use secondary compounds from the plant to defend themselves against parasitism by *D. morosus*. So if *Barbarea* provides enemy free space to flea beetle larvae, it cannot be explained by any of these two mechanisms.

The finding that parasitism was higher on *Barbarea* than on *Sinapis* on 16 July and 23 July might be explained by the fact that at these dates the population of flea beetle larvae was bigger on *Barbarea* than on *Sinapis*. Rate of parasitism can be related to host density. When densities are higher, hosts are easier to find and closer to each other; the parasitoids show a functional response to this increased host density.

In Kværkeby it was found that early in the season parasitism was higher on *Sinapis* than on *Barbarea*. Unfortunately no data about density of larvae in this area is present, what makes it hard to say anything about the reason of the difference in parasitism on the two plant species. However, late in the season parasitism was really high on both plant species, which again indicates that *D. morosus* females are able to locate hosts on *Barbarea*.

Now we have arrived at the most interesting question: Are parasitism levels on *Barbarea* in general lower than those on *Sinapis* during the whole season? A straightforward answer to this question can not be given. However, the results from this study indicate that indeed parasitism is lower on *Barbarea* than on *Sinapis* if you look at the whole season. Overall parasitism rate of the whole season in Ejby, calculated without biomass data, on *Sinapis* is at least approximately 10% higher than on *Barbarea*. When biomass is incorporated in the calculations, the difference is even bigger: parasitism on *Sinapis* is then 15% higher than on *Barbarea*. Relatively, overall parasitism rate on *Sinapis* in that case is approximately 180% of the overall rate on *Barbarea*.

Interesting to see is that for both calculations, the one with and the one without biomass incorporated, the parasitism rate of *A. brevicauda* is around 5%. Including biomass in the calculations does not have much effect on the parasitism rate of this parasitoid. However, for *D. morosus* including biomass does have a clear effect: with biomass included the overall parasitism rate of *D. morosus* on *Sinapis* is 5% higher. The parasitism rate of this parasitoid on *Barbarea* does not change that much when biomass is included, there is only a slight decrease of 0.4%.

The relatively large increase of overall seasonal parasitism rate by *D. morosus* on *Sinapis* when biomass is included can be explained by the fact that biomass on the last collection date (7 july) is relatively high for *Sinapis*. Therefore still a lot of food for flea beetle larvae is present. This combined with the relatively high density on larvae on this date, make that on this date still a relatively large percentage of the total population of flea beetle larvae is present. The high parasitism rate of *D. morosus* on this last collection date therefore has a large effect on the overall seasonal parasitism rate on *Sinapis*.

On *Barbarea* the density of flea beetle larvae and the available biomass are relatively low on the last collection date, the date with by far the highest parasitism rate. So what can be seen from these results is that the population of larvae on *Barbarea*, by peaking earlier in the season, can for a big part escape the really high parasitism levels found at the end of the season. The population of larvae on *Sinapis* is not able to do this, and therefore in the end suffers from a higher overall seasonal parasitism rate.

### 4.2. Enemy free space

So does *Barbarea* really provide enemy free space to flea beetle larvae? As said in the introduction of this research, to answer this question positively 3 hypotheses should be tested (Berdegue et al. 1996). These hypotheses were:

1. the fitness of the organism in the original habitat with natural enemies should be less than the fitness of the organism in the original habitat without natural enemies;
2. the fitness of the organism in the alternative habitat with natural enemies should be greater than the fitness of the organism in the original habitat with natural enemies, and;

3. the fitness of the organism in the alternative habitat without natural enemies should be less than the fitness of the organism in the original habitat without natural enemies.

The first hypothesis is true, because extremely high parasitism rates of more than 90% were found. The third hypothesis is true because *Barbarea* is a less suitable host plan than *Sinapis* (Nielsen 1999, de Jong and Nielsen 2002). In this research it is found that also hypothesis 2 is true. Parasitism levels on *Barbarea* are, when looking at the entire season, lower than parasitism levels on *Sinapis*. Therefore, it seems that indeed *Barbarea* provides enemy free space to larvae of *P. nemorum*.

In this case study there are two mechanisms behind the enemy free space. Firstly, this year no larvae were attacked by *A. brevicauda* on *Barbarea*, while parasitism levels of *A. brevicauda* could reach levels of approximately 15 % (in Amager) on *Sinapis*. Secondly, because flea beetle larvae peaked earlier on *Barbarea* than on *Sinapis*, the population of larvae on *Barbarea* escaped from the extremely high parasitism levels caused by *D. morosus* at the end of the season.

Selection pressure from generalist parasitoids most probably restricts the host range of phytophagous insects, while selection pressure from specialist parasitoids most likely broadens this host range (Mulatu et al. 2004). Both parasitoid species in this research are not restricted to *P. nemorum*, but their host range is limited (Alford 2003). Therefore it is more likely that selection pressure from both parasitoid species results in a host range expansion for *P. nemorum*. This is also what happened in East Denmark: flea beetles still use their old hosts, but a part of the population in addition can also use a new host, *Barbarea*. However, whether the enemy free space is really the driving force behind this host range expansion is not 100% sure. Other factors, such as plant chemistry and a number of ecological variables also play a role in host selection (Jaenike 1990).

### 4.3. The effect of temporal variation

As mentioned in the introduction, larvae of the apple maggot fly *Rhagoletis pomonella* (Diptera: Tephritidae) feeding on apples suffered from lower mortality by parasitoids than larvae of this species feeding on hawthorn. One reason for this difference was that the apples had an earlier fruiting phenology than the hawthorns (Feder 1995, Feder and Filchak 1999). This enabled the larvae of the apple maggot fly to develop earlier in the season, when the populations of natural enemies were still relatively low (Feder 1995). This is also a kind of enemy free space which is related to phenology of the insects and the plants. So the idea that hosts can escape their natural enemies because of asynchrony in phenology of the host and the parasitoids is not new. However, what is interesting about the case study in East Denmark is that, if you look at each separate collection date, parasitism is never really lower on the alternative host than on the old host. Still in the end it turns out that selection pressure of parasitism, looking at the whole season, is lower on the alternative host than on the old host. This finding indicates that just parasitism levels at a certain moment do not really say anything about the total impact of this parasitism on the population. It has to be known which part of the population is present at the time of the measurement of the parasitism level. Therefore, if in the future similar experiments are done, it is essential that data about the number of hosts are collected simultaneously with collecting the data about parasitism level.

Within and between season variation of the impact of parasitoids can be high in natural systems (Scheirs and De Bruyn 2002). Also in this study parasitism rate varied considerably during the season. This finding shows that, if the impact of a parasitoid on its host is investigated, it is essential that the entire season is monitored on a regular basis. For

example, just comparing early parasitism and late parasitism is not sufficient to draw real conclusions about the impact of a parasitoid on the population of its host.

Variation between seasons can also be high, and therefore studies spanning more than one season are more reliable than studies of just one season (Scheirs and De Bruyn 2002). Unfortunately, this study only spans one year. However, some data is present about parasitism rate of earlier seasons (de Jong and Nielsen, unpublished data). It was found that in the summers of 1999 and 2000, early in the season parasitism rate was significantly lower on *Barbarea* than on *Sinapis*. This was found both in Ejby and in Kværkeby. If in these years also *Barbarea* flowered and developed earlier in the season than *Sinapis*, which was observed but not measured quantitatively, the difference between overall seasonal parasitism levels of larvae on *Barbarea* and *Sinapis* could have been even bigger than found in this year's research. This might indicate that at the time of the development of the resistance to the defence mechanisms of *Barbarea*, the relative advantage of using *Barbarea* instead of *Sinapis* as a host plant was bigger than now.

An explanation for the fact that in 1999 and 2000 in Ejby the parasitism levels early in the season on *Barbarea* were lower than on *Sinapis* and in 2006 they were not, might be that the population of flea beetle larvae on *Barbarea* in 1999 and 2000 was smaller than in 2006. As said before, when densities are larger, larvae are easier to find and closer to each other, allowing the parasitoids to reach higher levels of parasitism. Perhaps since 2000 the population of flea beetles on the new host *Barbarea* has increased.

In this year's research it was found that in Kværkeby parasitism early in the season was significantly lower on *Barbarea* than on *Sinapis*. So probably also in Kværkeby, where *Barbarea* definitely developed earlier in the season than *Sinapis*, the difference in parasitism rates on the two plant species was higher than in Ejby.

### 4.4. *Diospilus morosus*

This year the most important natural enemy of flea beetle larvae was *Diospilus morosus*. It was found that this species is also the most common parasitoid of *P. nemorum* in areas in France, Germany, Switzerland and the UK (Alford 2003). This species can have up to four generations in one year. For overwintering, *D. morosus* switches to larvae of cabbage stem flea beetles at the beginning of September (Alford 2003). Because of a poor synchrony of the last generation of *D. morosus* and the larvae of cabbage stem flea beetles at the end of the season, the first generation of *D. morosus* the next year usually is very small (Alford 2003). The second generation is usually more abundant than the first (Alford 2003), just like was found in this research. Parasitism levels up to 90% were found (Alford 2003).

In this research only a first and a second generation of *D. morosus* was found. It seems like it is impossible for this parasitoid to have a third generation on flea beetle larvae feeding on *Barbarea*. The density of flea beetle larvae on *Barbarea* declined gradually after it peaked, and was really zero or very close to zero the last collection date. However, it might be possible that these wasps can have a third generation on flea beetle larvae feeding on *Sinapis*. As said before, the *Sinapis* plot in Ejby was mown before the end of the research. I explained before that I do not think it had a significant effect on the densities and the parasitism levels measured in this area until 7 July. However, it might be possible that the mowing had an effect on the measurement on the last collection date, done on 16 July. Maybe when no mowing had been done, more adult flea beetles would have been still in this *Sinapis* site several days before this collection date (as said, the situation on 7 July for the first time showed the negative results of the mowing), which could have had the effect that more flea

beetle larvae would have been present on 16 July. It is possible that this would have enabled the population of *D. morosus* to have a third generation.

It is not easy to say what the consequence of this possibility is. Most likely the availability of larvae on *Sinapis* later in the season would only have made the difference between parasitism rate of larvae on *Barbarea* and larvae of *Sinapis* bigger. Parasitism on 7 July was extremely high, so probably on 16 July parasitism would have been quite high as well. If parasitism on that date was higher than the 34.1% calculated as the overall parasitism rate of the entire season on *Sinapis*, then overall parasitism on *Sinapis* would only have increased. However, it is not sure how parasitism levels would have developed in reality.

## 4.5. *Aneuclis brevicauda*

That in this research it was found that *D. morosus*, and not *A. brevicauda*, was the most important natural enemy of *P. nemorum* was not completely unexpected. In literature it is already stated that, compared with *D. morosus*, *A. brevicauda* is thought to be less effective at regulating the density of *P. nemorum* (Alford 2003). In earlier researches done in Germany and Switzerland, it turned out that the parasitism rate was only 0.1-5% at half of the sampling sites, although maximum parasitism was 22% at one site (Alford 2003).

Just like *D. morosus*, *A. brevicauda* needs other hosts than *P.nemorum* to overwinter (Alford 2003). However, these hosts have not yet been identified (J.K. Nielsen, personal communication).

### *4.5.1. Behavioural preference*

The fact that during this research *A. brevicauda* was only found on *Sinapis*, and not on *Barbarea*, is a very interesting finding. An explanation for the lower parasitism rates could be that *A. brevicauda* shows a behavioural preference for *Sinapis*. To see how this is possible first we need to know how parasitoid wasps find their hosts.

Many insect parasitoids seem to rely on a series of physical and chemical cues to find their hosts (Vinson 1976, Vet and Dicke 1992). Sources of chemical cues can originate from the herbivore host itself, from the plant it is feeding on, or from the interactions between the herbivore and the plant (Vet and Dicke 1992). Long-range chemical cues emitted by plants play a crucial role in host location by foraging female parasitoids (Guerrieri et al. 1999). The plant often is the first cue used by the wasp to locate its host (Vinson 1976). This is because chemical cues emitted by plant are relatively easy to detect, because of the plant's relatively large biomass (Vet and Dicke 1992). However, chemical cues emitted by plants are only reliable if infestation rates on the plant are high (Vet and Dicke 1992). Chemical cues emitted by the herbivore itself, by for example its faeces, cuticle or pheromones, on the other hand are very reliable, but not so abundant. The chemical information emitted by the herbivore host itself becomes more important the closer the seeking parasitoid is coming to its host (Vinson 1976, Vet and Dicke 1992).

It seems that information from only the plants or only the herbivore itself does not seem to be the ideal way of finding a host, because of respectively the low reliability and the low detectability. When herbivores feed on plants, this herbivory induces characteristic plant volatiles, which can be used by natural enemies for host detection (Guerrieri et al. 1999). These plant volatiles are called herbivore-induced synomones (Vet and Dicke 1992). Synomones are a class of infochemicals that are favourable for both the emitter and the receiver. Herbivore-induced synomones can be easy to detect because when a certain

herbivore is feeding on a plant the emission of this chemical blend is not restricted to the damaged area, but is released systematically by the whole plant under attack. Furthermore it is reliable, because in most cases only plants on which the herbivore is feeding are releasing the synomone (Vet and Dicke 1992).

Because of these herbivore-induced synomones, chemical cues emitted by damaged plants of a certain species differ from cues emitted by undamaged plants of the same species. There is also a difference between emitted cues of damaged plants of different species. Variation in cues among plant species and cultivars can be greater than between damaged and undamaged plants of the same species. These differences can be reflected in the attraction of parasitoids to certain plant species (Bukovinszky et al. 2005).

### *4.5.2. Chemical differences between* Barbarea *and* Sinapis

Glucosinolates and mustard oils (isothiocyanates) are often used by specialist crucifer feeders such as flea beetles as positive cues for host plant recognition (Renwick 2002). Isothiocyanates are among the products of the hydrolysis, in the presence of the enzyme myrosinase, of glucosinolates (Renwick 2002). The isothiocyanates are often volatile, while the glucosinolates are not. It would be interesting to see if there is a difference in glucosinolate, and thus isothiocyanate, content of *Barbarea* and *Sinapis*, because a possible difference could explain the behavioural preference of *A. brevicauda* for one of the two plants.

Griffiths et al. (2001) identified the glucosinolates on the leaf surface of several crucifers, including our species of interest. Glucosinolates on the surface of crucifers are qualitatively similar to those inside the leaves (Griffiths et al. 2001). Glucosinolates found on *Sinapis* are 8-methylsulfonyloctyl, 9-methylsulfonyloctyl and sinalbin. Glucosinolates found on *Barbarea* are glucobarbarin, gluconasturtiin, glucobrassicin and 4-methoxyglucobrassicin.

Unfortunately the molecular weight of all these glucosinolates is large, and the hydrolysis of these glucosinolates will only produce non-volatile isothiocyanates (N. Agerbirk, personal communication). Therefore a possible behavioural preference for one of the plant species most likely cannot be explained by differences in glucosinolates or isothiocyanates produced by the plants.

However, studies using electrophysiological techniques have shown that there are several other compounds that play a role in host selection (Renwick 2002). Many components that can play a role in host finding by parasitoid wasps still need to be identified (Renwick 2002). So although a behavioural preference of *A. brevicauda* for one of the two species probably can not be explained by differences in glucosinolate content, there are still other (chemical) possibilities for the parasitoid wasp to distinguish between the two species.

### *4.5.3. Food and colour*

Many insects, including hymenopteran parasitoid wasps, are able to distinguish between colours (Wackers 1994, Wackers and Lewis 1994, Shafir 1996, Oliai and King 2000). Wackers (1994) found that food-deprived parasitoids of the species *Cotesia rubecula* (Hymenoptera: Braconidae) seek out yellow targets. Most parasitoid adults require food as an energy source for flight and/or the production and maturation of eggs (Wackers 1994). So there is a good possibility that also adults of *A. brevicauda* require food. The flowers of *Barbarea* and *Sinapis*, which are both yellow, are potential food sources for the wasps. At the time that *A. brevicauda* parasitism was found to be highest in this research, the *Barbarea*

plants already stopped flowering. In contrary, large numbers of *Sinapis* plants were still flowering, and thus yellow. Therefore it might be possible that the difference in parasitism between *Sinapis* and *Barbarea* is caused by the fact that adults of *A. brevicauda* are attracted to the available food, or the yellow flowers, of *Sinapis*.

### *4.5.4. Preference experiments with* A. brevicauda

It would be interesting to test whether adults of *A. brevicauda* really have a behavioural preference for *Sinapis*, and if so, what mechanism causes the preference. There are basically three ways to test if the preference is caused by chemical differences between the plant species: 1) choice wind-tunnel bioassay (Rodriguez-Saona et al. 2005); 2) no-choice wind-tunnel bioassay (Guerrieri et al. 1999) and 3) Y-tube bioassay (Storeck et al. 2000, Bukovinszky et al. 2005). At Wageningen University there is a good opportunity to do Y-tube bioassays.

To test if a possible difference is caused by attraction of the colour yellow, the method described by Oliai and King (2000) could be used. It is often found that colour preference is induced by associative learning (Wackers and Lewis 1994, Shafir 1996, Oliai and King 2000), so this aspect should also be taken into account.

### *4.5.5. High seasonal overlap*

The seasonal overlap calculated for *A. brevicauda* and flea beetle larvae on *Sinapis* is 92.4%, which is very high. This might indicate that *A. brevicauda* synchronized its lifecycle to that of flea beetle larvae on their old hosts, which could mean that flea beetle larvae are the main host of *A. brevicauda*.

## 4.6. Weaknesses of this research

Like all ecological field studies, also this one is not perfect. Firstly, I only looked at natural enemies of *P. nemorum* in their larval stage. However, it is likely that flea beetles are also attacked by natural enemies in their other life stages (egg, pupa and adult). Secondly, there is the fact that one of the most important study areas, the *Sinapis* field in Ejby, was mown before the end of the research. However, as I explained earlier, I do not think it had much effect on the outcome of this research. Of course it would have been better if the mowing had not happened. Third, some assumptions had to be made to calculate the overall parasitism rate of the whole season. It was assumed that the number of plants stays the same during the larval season. This assumption is probably a good one, because by the time the flea beetle larvae were present the plants were already of such a size that they would not easily disappear. Almost no new seedlings were observed during the larval season.

Furthermore it was assumed that the biomass is proportionally related to the total number of leaves. Most probably this is not entirely true. It is true that biomass increases when more leaves develop, however biomass also increases when the already existing leaves grow bigger. This effect is not taken into account. Therefore it is expected that the number of leaves does not change as much in time as the biomass. However, there certainly is a relation between number of leaves and biomass. Because there is no data about number of leaves, but only about biomass, I chose to use biomass as an indication for number of leaves.

It must be noted that even when biomass is not taken into account, still the overall parasitism rate calculated for larvae on *Sinapis* is higher than the rate for larvae on *Barbarea*.

One could say that also the density of larvae is related to the size of the leaves. The bigger the leaves, the more larvae can feed on it. However, during the larval season the leaves on average are big enough to contain more larvae than they do in reality. Therefore I think it is all right to assume that density is not related to leaf size during the larval season.

Because there could have been a third generation of *D. morosus* on *Sinapis* in Ejby if the area had not been mown, and because in earlier years (1999 and 2000) in Ejby, and this year in other areas (Kværkeby), parasitism early in the season was lower on *Barbarea* than on *Sinapis*, and because the biomass of the *Barbarea* plants in reality decreased faster after the flowering peak than was calculated, I think the estimates I made concerning overall seasonal parasitism rate are on the conservative site. All in all I believe that it is safe to conclude that *Barbare*a really provides enemy free space to flea beetle larvae, by means of the two earlier described mechanisms.

### 4.7. Seasonal variability in defences of *Barbarea*

In this research some evidence is found that indeed adult flea beetles which are not resistant to *Barbarea* do use this plant early in the season. However, more research has to be done before it is safe to say anything about this. The area where the susceptible adults were found on *Barbarea*, Amager, has just recently been invaded by *P. nemorum* (J.K. Nielsen personal communication). Newly invaded areas are most probably the most suitable areas to investigate this research question.

### 4.8. Experimental plots

Although some data could be obtained from the experimental plots, I think it would be very interesting to give the experiment another try. No data about density of larvae or parasitism early in the season was collected this year in the plots, because early in the season the number and size of plants in the plots was too small. Another disadvantage of the method used here is that the time of sowing influenced the time of germination and the whole phenology of the plants. For this research, it is necessary that the plants in the plot develop simultaneously with their wild equivalents in the area. Therefore next time seeds need to be sown inside the plots several weeks before the conditions favour germination, making sure that the seeds germinate at the same time as the wild *Barbarea* and *Sinapis* in the vicinity.

### 4.9. Summarizing the conclusions

The most important conclusions that can be drawn from this research are:
- *Barbarea* grows and develops earlier in the season than *Sinapis* in East Denmark;
- larvae of *P. nemorum* therefore peak earlier in the season on *Barbarea* than on *Sinapis*;
- larvae on *Barbarea* can therefore escape from the extremely high parasitism rates, caused by *D. morosus*, at the end of the season, while larvae on *Sinapis* can not;
- larvae on *Barbarea*, compared to larvae on *Sinapis*, also suffer from less parasitism by *A. brevicauda*;
- these two mechanisms combined result in an enemy free space on *Barbarea*;
- this enemy free space might be the reason for the host range expansion of *P. nemorum*.

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# Appendix

Included is a cd-rom with all relevant data. The “read me” file on the disc explains where to find what data. Also some photos of the field work, the lab work and the insects and plants included in this study can be found on the cd-rom.