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Detection of refuge from enemies through phenological mismatching in multitrophic interactions requires season-wide estimation of host abundance

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Abstract The concept of "enemy-free space" (EFS) refers to ways of living that reduce or eliminate the vulnerability of a species to natural enemies. It has been invoked to explain host shifts of phytophagous insects. A demonstrated cause of EFS is escape from enemies in time, through phenological mismatching of herbivore development and enemy occurrence, leading to low percentages of predation/parasitism of herbivores occurring at a certain time. The mere measurement of percentage parasitism, however, is not sufficient to demonstrate EFS in certain cases. Here we present such a case, where parasitism was studied of a phytophagous insect (*Phyllotreta nemorum*), using two different host plant species in the field: an atypical, relatively rarely used, plant (*Barbarea vulgaris*), and a more widely used one (*Sinapis arvensis*). At one location we found a paradoxical result: on each separate sampling day throughout the season the percentage of parasitism of *P. nemorum* using a patch of *B. vulgaris* was not significantly different from, or even significantly higher than on a nearby patch of *S. arvensis*. The overall season-wide proportion parasitism of the flea beetle cohort using the *B. vulgaris* patch, however, was lower. We conclude that, in the year and at the location we studied, the patch of *B. vulgaris* provided enemy-free space to the herbivore in the form of a temporal refuge, and that the importance of enemy-free space in the use of an atypical host plant should be evaluated on the basis of season-wide sampling, including estimation of host population size.

Keywords Enemy-free space · Parasitism · Temporal variation · Flea beetles · Asynchrony in phenology

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Introduction

Enemy-free space

Originally defined by Jeffries and Lawton in 1984, but implicitly already considered earlier (e.g. Price et al. 1980), enemy free space involves 'ways of living that reduce or eliminate species vulnerability to one or more species of natural enemies'. As such, it has been invoked repeatedly as a selective factor influencing the host-plant range, and thereby diversification, of phytophagous insects (e.g. Bernays and Graham 1988; Ohsaki and Sato 1994; Singer and Stireman 2005). Many phytophagous insects are specialized in their use of host plants (Ehrlich and Murphy 1988; Stamp 2001). These specialized insects are expected to use the host plants on which their performance is optimal, i.e. maximized given constraints. Some herbivores indeed show a positive correlation between adult oviposition preference and larval performance (Denno et al. 1990). It has also been frequently found, however, that herbivorous insects use plants or plant parts on which larval performance is sub-optimal (Gratton and Welter 1999; Mulatu et al. 2004). Insects on these apparently sub-optimal host plants may be able to escape from competitors, predators, or parasitoids (Mulatu et al. 2004), the latter being a frequent cause of death for many immature insect herbivores (e.g. Hawkins et al. 1997). Ohsaki and Sato (1994) showed that food plant preferences of three *Pieris* species results from the balance of a trade-off between parasitoid avoidance and the quality of the plants as food source. Seemingly sub-optimal plants may provide enemy-free space to herbivorous insects, thereby enhancing their fitness.

Dozens of examples of studies involving enemy-free space can be found in the literature (Ohsaki and Sato 1994; Berdegue et al. 1996; Scheirs and De Bruyn 2002; Murphy 2004; Heard et al. 2006). Amongst the mechanisms generating enemy-free space, asynchrony in seasonal distribution of natural enemies and their hosts (Feder 1995) forms a prominent example. 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 fruited earlier 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 densities of natural enemies are still relatively low (Feder 1995). The work on *Rhagoletis* has inspired much other work on parasitism of phytophagous insects using different host plants (see for review Heard et al. 2006), with variable success in demonstrating the existence of EFS. Typically, in such studies, the percentage of parasitism (often confusingly called "rate" of parasitism) of hosts living on different host plants is compared. In some recent work, for example, phytophagous insects were reared in the laboratory, put out into the field on different host plants, after which survival over a certain period of time was determined (e.g. Mulatu et al. 2004; Murphy 2004; Ishihara and Ohgushi 2008; Wiklund and Friberg 2008). Whereas such an approach is valid under certain conditions, caution is required when the factors influencing the proportion of hosts being parasitized are not constant through time, e.g. when phenology may play a role. Imagine, for example, that parasitoid-density is gradually increasing in the course of the season. Hosts are present early in the season on plant A, and later in the season on plant B. Parasitism on plant A is *higher* than on plant B for every single moment during the season. With this scenario, it is theoretically possible that the overall proportion of the cohort of hosts on plant A that is parasitized over the season is *lower* than on plant B (Fig. 1). Only knowing the percentage of parasitism on a certain date(s) is often not sufficient to imply the impact parasitoids have on an entire population

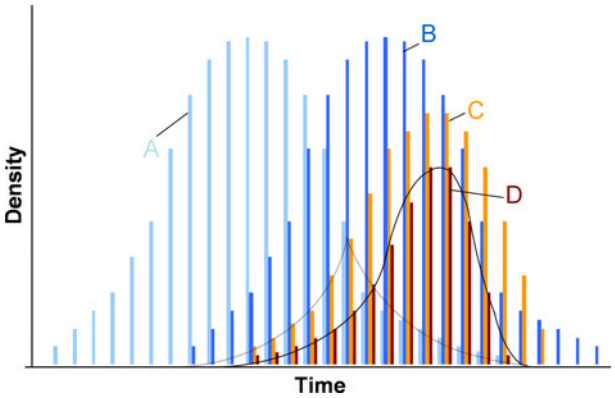

Fig. 1 Schematic diagram illustrating the discrepancy between actual parasitism at any one moment and overall parasitism across the whole season. The *light blue bars* (*A*) represent the density of flea beetle larvae on *Barbarea* at various times through the season, the *dark blue bars* (*B*) that on *Sinapis*; the peak of larval density on *Sinapis* is lagging behind that on *Barbarea*. The *orange bars* (*C*) show the risk of parasitism of flea beetle larvae on *Barbarea* through time, while the *brown bars* (*D*) represent parasitism risk on *Sinapis*, the latter being assumed to be lower than the former at any specific time to illustrate our argument. The overlap between the *light blue* and the *orange graph* (area under *stippled line*), and between the *dark blue* and *brown graph* (area under *solid line*), respectively, indicates the proportion of the cohort of flea beetle larvae on *Barbarea* and *Sinapis* that is parasitized over the whole season. Whereas parasitism on *Barbarea* is higher than on *Sinapis* at any particular moment (*orange bars* higher than *brown ones*), the parasitism of the cohort of flea beetles on *Barbarea* across the whole season is lower than on *Sinapis*

of hosts at a certain location. The percentage of parasitism in one of the tails of the seasonal dynamics of population size will only marginally contribute to the overall chance to become parasitized, whereas parasitism at the peak of the population size will have a major impact on the overall parasitism level. It is the overall proportion of the host-population using a certain host plant that is parasitized, that determines the fitness of individuals using that host plant, and thus the evolution of host plant use.

Case study in Denmark

A case study for which we suspected that phenological differences and associated parasitism risks might influence host plant use is that of the flea beetle *Phyllotreta nemorum* L. (Coleoptera: Chrysomelidae: Alticinae) and its host plants. *P. nemorum* is a pest on various crucifer species, both crop plants (Ulber and Williams 2003), and also several wild crucifers (Nielsen 1977). One of these wild host plants, *Barbarea vulgaris* ssp. *arcuata* (Opiz.) Simkovics, is genetically polymorphic with respect to its chemical defence to herbivorous insects. Two genotypes have been distinguished, of which one produces saponins, which have been shown to be the biologically active compound protecting the plant against phytophagy (Shinoda et al. 2002; Nielsen et al. 2010a, b). Both genotypes occur naturally in the field, where approximately 75% of the known Danish populations are of the chemically defended genotype (called the 'G-type', with glabrous leaves), 20% are of the genotype that is not chemically defended ('P-type', with pubescent leaves), and approximately 5% are hybrids between these, or unclassifiable (J. K. Nielsen, unpublished data). Of the flea beetles, also different genotypes have been found: susceptible and resistant to the defences of *B. vulgaris*, which can not, and can, respectively, use the defended genotype of this plant (henceforth indicated by the shorthand '*Barbarea*') as food (de Jong and Nielsen 1999;

de Jong et al. 2000; de Jong and Nielsen 2002; Renwick 2002). Resistant flea beetles are relatively rare, which has led to the hypothesis that the use of *Barbarea* represents a host-range expansion of *P. nemorum* (Nielsen 1997). The genotype of *B. vulgaris* ssp. *arcuata* that is not chemically defended is rarely used as host plant by *P. nemorum*. The most abundant and widely used host plants of *P. nemorum* in Denmark are charlock (*Sinapis arvensis*) and radish (*Raphanus raphanistrum*). *Cardaria draba* is used by a few coastal populations of *P. nemorum*. Cultivated radish and turnip (*Brassica rapa* ssp. *rapa*, which is currently rarely grown) are also used as host plant. No information is available on the use of wild *Brassica rapa*, which has a western distribution in Denmark, and is rather common on sandy soils. *Brassica rapa* ssp. *oleifera* is not accepted as host plant, and neither is the major crop *Brassica napus* (oilseed rape) (Nielsen 1977 and unpublished results).

Amongst the results of earlier and ongoing studies (De Jong and Nielsen 1999, 2000, 2002; de Jong et al. 2000; Nielsen and De Jong 2005), one in particular has prompted us to initiate the work reported in this paper: the use of defended *Barbarea* as food by the resistant flea beetles clearly involved physiological costs. When the resistant flea beetle larvae used defended *Barbarea* as food, they developed slower than on other plants, including *S. arvensis* (Nielsen 1999). Moreover, homozygous resistant flea beetles appeared to suffer from high mortality on different plant species in laboratory experiments (de Jong and Nielsen 2000). These results have led to the question: why do the flea beetles use the defended *Barbarea* at all, i.e. how has the resistant genotype of the flea beetles evolved? One of the factors that are likely to contribute to the use of this atypical host plant is predation pressure. If the use of *Barbarea* reduces the risk of the flea beetles to become parasitized/predated at certain times and/or localities, this factor may influence the use of *Barbarea* as host plant by counteracting any reported negative effects of this plant, i.e. *Barbarea* may provide enemy-free space to the flea beetles.

P. nemorum has one generation per year in Denmark, and adults overwinter in diapause in the soil. Eggs are laid in the soil close to the host plants, and newborn larvae climb into a nearby plant, after which they start mining its leaves (Nielsen 1997). The larvae of *P. nemorum* are known to be attacked by at least five hymenopteran parasitoids (Ulber and Williams 2003), including *Diospilus morosus* Reinhardt (Braconidae) and *Aneuclis brevicauda* Thomson (Ichneumonidae). *D. morosus* is a solitary endoparasitoid, which has been described as a multivoltine species in the areas where their phenology was studied (France and S.-W. Germany/W. Switzerland; see Ulber and Williams 2003 and references therein). *D. morosus* has been shown to switch host, and apart from *P. nemorum* also parasitizes larvae of *Ceutorhynchus assimilis* (Paykull) and *Psylliodes chrysocephala* (L.). When *D. morosus* parasitizes larvae of *P. nemorum*, their larval development is only completed when the *P. nemorum* larvae have dug into the soil for pupation. *A. brevicauda* has also been described as a solitary, multivoltine endoparasitoid, and appears to switch to another host than *P. nemorum* for overwintering, although the latter has not been documented (Ulber and Williams 2003). In the part of Denmark where *Barbarea*-resistant flea beetles are found, parasitism levels of flea beetle larvae of more than 60% (and up to nearly 100%) have been found, but the observed percentages of parasitism were very variable across host plants, sampling time and locations (P. W. de Jong and J. K. Nielsen, unpublished data). It has been found that *Barbarea* grows and flowers earlier in the season than alternative host plants of *P. nemorum* in Denmark (J. K. Nielsen, P. W. de Jong, personal observation), corresponding with earlier observations on this species in the USA (Root and Tahvanainen 1969). If this plant can therefore be colonised earlier in the season by *P. nemorum*, and if this affects synchronisation with populations of parasitoids, this difference in host plant phenology may provide enemy-free space to the flea beetles. The role of enemy-free space,

in this case, is likely to vary with time and location, depending on the precise timing of plant-growth, recruitment by flea beetles, and parasitoid density.

To test for enemy-free space, three conditions as formulated by Berdegue et al. (1996) should be addressed. The first condition tests the importance of natural enemies in the system. The second one tests whether mortality caused by natural enemies is less on the new or atypical host plant. The third condition that needs to be verified is whether there is a cost of feeding on the new host plant. The verification of this third condition is necessary to be able to conclude that escape from natural enemies is indeed the main driving force behind the host switch or -extension (Mulatu et al. 2004). Although no conclusive proof is available as yet, there is some evidence that the first and third condition may apply to the flea beetle system. Because high levels of parasitism of *P. nemorum* were found on commonly used host plants such as *Sinapis arvensis* and *Raphanus* spp. (P. W. de Jong and J. K. Nielsen, unpublished data), and parasitoids kill their host by parasitizing them, it is likely that parasitism indeed reduces fitness of the flea beetles, which would satisfy condition one. *Barbarea* has also been found to be a less profitable host plant compared to the alternative host plants (in agreement with condition three; Nielsen 1999; de Jong and Nielsen 2000); this was indeed the incentive for the present study (see above). In this research the remaining condition formulated by Berdegue et al. (1996) was addressed, namely whether mortality caused by natural enemies is lower on *Barbarea* than on *Sinapis,* and whether this is related to the difference in phenology of the different host plants. We explicitly studied the possibility of enemy-free space by not only determining the percentage parasitism of host samples collected throughout the season, but by also estimating the host population dynamics over the period of sampling to enable the calculation of a season-wide risk of parasitism of flea beetle larvae using different host plants as food.

Methods

Timing of the study

This research was carried out from the beginning of April 2006 until the end of July 2006, covering the whole reproductive season of the flea beetles. Larvae of *P. nemorum* had, in earlier observations at the same localities, been found to be parasitized by *A. brevicauda* from the end of May to the end of July (see also Ulber and Williams 2003).

The host plants

Sinapis arvensis L. (henceforth indicated by '*Sinapis*') is the most common host plant of flea beetles in East Denmark (J. K. Nielsen, personal communication). In this study, *Sinapis* was selected as representative of typical host plants of the flea beetles (other plants where they can be commonly found are *Raphanus* spp.; J. K. Nielsen, unpublished results). It is an annual (Gols et al. 2008), in contrast to *Barbarea*, which can be an annual, biennial, or perennial (Macdonald and Cavers 1991). Thus, *Barbarea* can overwinter as a rosette, enabling its use by *P. nemorum* early in the season. The *Barbarea* sampled in the present study was of the chemically defended type only, which can be distinguished morphologically from the non-defended type by their glabrous leaves (as opposed to hairy ones in the not chemically defended *Barbarea*; see Kuzina et al. 2009). This type has been found to occur more commonly in Denmark than the undefended type (75% vs. 20%, with 5% unclassifiable specimens).

Study area

In East Denmark two well studied areas were selected where both *Sinapis* and *Barbarea*, resistant flea beetles and *A. brevicauda* had been present in earlier years: Ejby (N55°42′ by E12°25′) and Kværkeby (N55°28′ by E11°55′; Fig. 2). In Ejby, one *Barbarea* patch and one *Sinapis* patch were sampled. The distance between the two patches was approximately 350 m; this was the smallest distance that we could find between the two plant species. In the second location, Kværkeby, one *Sinapis*- and two *Barbarea* patches were sampled. The distances between the *Barbarea* patches and the *Sinapis* patch were approximately 1,000 and 1,650 m. The distance between the two *Barbarea* patches was about 2,500 m. Other patches of *Barbarea* and *Sinapis* were present in the vicinity of the studied plots at both locations, but these were not studied in detail. In the direct surroundings of the study sites, no other host plant species than the two studied ones could be found.

Additional research, mainly concerning phenological characteristics of the two plant species, was done in two other areas: Amager (N55°38′ by E12°34′) and Suserup (N55°25′ by E11°31′; Fig. 2). In Amager, only *Barbarea* occurred in our study area; in Suserup, the site only contained *Sinapis*.

Plant growth

Phenological characteristics of both *Barbarea* and *Sinapis* were measured from April–July at the four different locations. *Barbarea* growth was monitored in Amager, Ejby and Kværkeby, *Sinapis* characteristics were measured in Kværkeby and Suserup. In the areas, 1 × 1 meter plots were laid out (five per plant species per location) in such a way that the whole study area was represented as well as possible. In these plots the approximate height and diameter of the plants were estimated by defining size-classes (Table 1), and flowering was recorded.

For each height/diameter class combination (e.g. height class 1, diameter class 3) which was present in the field, five plants per species were taken to the laboratory to measure the biomass of the class combinations. The leaves of these plants were picked and dried in an oven, individually in paper bags, for at least 4 days at 70°C. After drying, the total dry

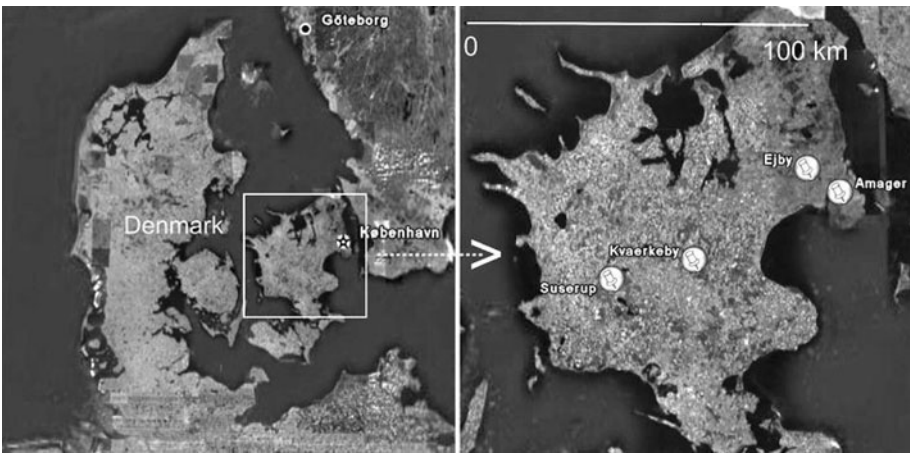

Fig. 2 The location of the four study areas of this research (Google Earth images)

Table 1 Definitions of size-classes used to score the height and diameter of host plants of *P. nemorum*

Class number	Height/ diameter (cm)
1	≤5
2	5.1–10
3	10.1–20
4	20.1–50
5	≥50

weight of the leaves of each plant separately was measured. The average total leaf-biomass of the five plants in each height-diameter combination was calculated. This was used to calculate the average biomass of the plants in a plot, and of the plants at a certain patch.

Plant data (height, diameter, flowering status) were first averaged within each of the 5 plots per plant species per patch. Subsequently, these values per plot were averaged for each patch and plant species. This yielded a mean proportion of flowering plants and mean biomass per plant for each separate patch, per plant species, per collection date.

The *Sinapis* data collected in Kværkeby and Suserup, as well as the *Barbarea* data collected in the two patches at Kværkeby, were always collected on the same day. Therefore, for each collection date the average of the values of these two locations/patches was calculated (the host plant phenologies at the different locations were similar; see Fig. 3, where standard deviations across the different locations are indicated with bars). The *Barbarea* data of Kværkeby, Ejby and Amager were collected on different dates. To yield an estimate 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 combined study areas in this week was calculated and allocated to the 4th day of the week.

For both the *Barbarea* and the *Sinapis* series, regression lines were calculated, relating the estimated plant variables to day of the year. The trend lines, either parabolic or logistic, were used to calculate peak dates. For the parabolic lines this is straightforward. The time to the peak for the logistic regression line was estimated as twice the time to the inflexion point of the growth curve.

Parasitism frequency

To determine the proportions of parasitized flea beetle larvae, approximately 100 leaves containing third (last) instar larvae per host plant species were picked in the field from as many different individual plants as possible, and brought to the laboratory, where they were kept in plastic bags at a temperature of 20–28°C. Thus, larvae were collected on 9 June, 15 June, 23 June, 30 June and 7 July in Ejby, and on 20 June and 12 July in Kværkeby. After June 15 on *Barbarea*, and after June 30 on *Sinapis*, larval densities started to diminish. On the 16th of July, the number of larvae in Ejby was negligible on both plant species, showing that the entire larval season had been monitored (*Phyllotreta* spp. have a single generation annually (Alford et al. 2003), and the phenology found in our study matches earlier findings (Nielsen 1977) as well as observations elsewhere (Ulber and Williams 2003)). Note that since only leaves containing larvae were collected, no absolute host density could be determined. We were, however, only interested in the relative density of hosts across the season (see below), and assumed that the number of larvae per larvae-containing leaf is correlated with larval density.

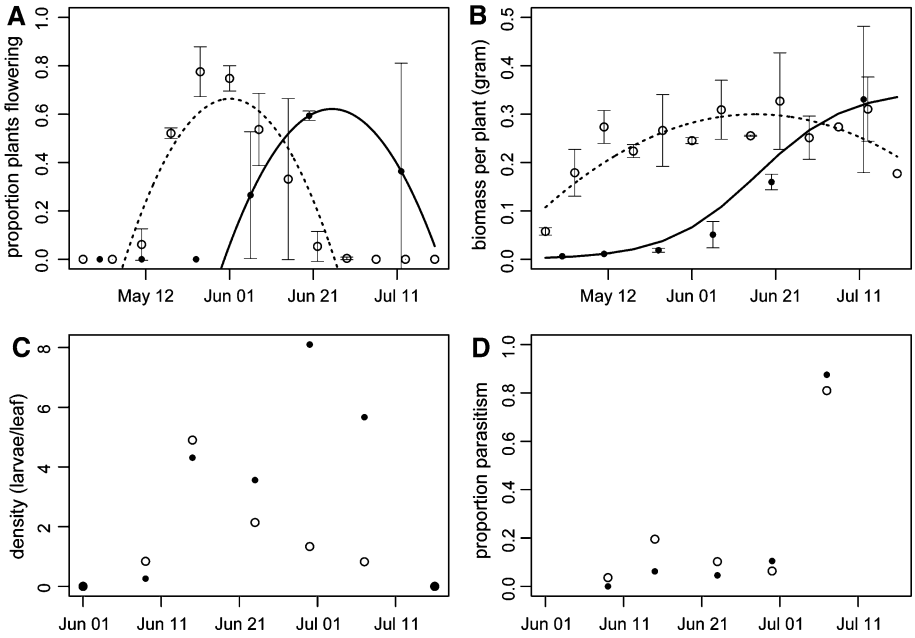

Fig. 3 **a** The proportion of flowering plants. The *open dots* represent the *Barbarea* data, the *black dots* the *Sinapis* data. This figure shows the data of all locations combined. *Error bars* represent standard deviation. *Trend lines* were calculated using non-linear regression. **b** Average biomass per plant. The *open dots* represent the *Barbarea*-, the *black dots* the *Sinapis* data. This graph shows the data of all locations combined. *Error bars* represent standard deviation. *Trend lines* were calculated using non-linear regression. **c** The average number of 3rd instar flea beetle larvae per leaf in Ejby on *Barbarea* (*open dots*) and *Sinapis* (*black dots*). **d** Overall proportions of parasitized 3rd instar flea beetle larvae on *Barbarea* (*open dots*) and *Sinapis* (*black dots*) in Ejby

Old and deteriorating leaves without larvae were removed from the bags daily. Bags containing *Barbarea* leaves were supplied with extra, laboratory reared chemically defended *Barbarea* leaves as food for the larvae to enable them to complete their development, even if the original leaves had wilted completely. Likewise, bags containing *Sinapis* leaves were supplied with supplementary chemically undefended *Barbarea* leaves (these lasted longer than *Sinapis* leaves, and are suitable for all beetle-genotypes).

Until the third day after collection, all bags were checked once or twice a day for final instar larvae that exit the mines to pupate. These larvae were counted and transferred to a jar (maximum 100 larvae/jar), where they could pupate. The jar contained a layer of mixed moist peat and medium grain vermiculite. Larvae from different host plants, locations and collection dates were kept separate.

To the jars a leaf was added (defended *Barbarea* for larvae from *Barbarea*, undefended *Barbarea* for larvae from *Sinapis*) to enable those final instar larvae that still needed some additional feeding before pupation to complete their development (undefended *Barbarea* was used rather than *Sinapis* for this additional feeding, because the *Barbarea* leaves remain fresh much longer than *Sinapis* leaves). 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 (the parasitoids kill their host only after the larvae have dug in to pupate). These beetles and parasitoids were

removed and counted, and parasitoids were kept for species determination. Jars were monitored until no more flea beetle adults and/or parasitoids had emerged for at least 2 weeks.

The number of parasitoids which emerged from a sample was divided by the total number of parasitoids and flea beetles emerging from that sample to calculate the proportion of parasitized larvae. It was assumed that external mortality (i.e. due to causes other than parasitism) of non-parasitized and parasitized beetle-larvae 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. Mortality (i.e. larvae that were unaccounted for) ranged from 0% to 65%. The highest mortality was found amongst larvae feeding on *Sinapis*, probably because *Sinapis* leaves deteriorate faster than *Barbarea* leaves, and become moist and sticky, trapping larvae even within their leaf mines (N. A. G. Kerstes and P. W. de Jong, personal observation).

In Ejby, the exact number of leaves that were collected had been recorded. This was used to calculate the relative density of third instar larvae in the field by dividing the number of third instar larvae by the number of collected leaves.

To estimate season-wide parasitism, parasitism on each sampling date needs to be weighed by individual sample sizes, since parasitism is not constant throughout the season. Moreover, to estimate the impact of parasitism on seasonal cohorts of flea beetles on different host plants, population-sizes at each collection day need to be estimated and corrected for. Since estimating the absolute number of larvae in the field is impossible, a method to obtain an estimate of the relative number of larvae in the field per collection date was developed as follows. An approximation for larval density was made: the number of third instar larvae per leaf collected in the field (d_i for density). An approximation for the total leaf biomass in the field is the average biomass of the plants in the field (b_i for biomass). b_i is obtained using the regression lines calculated for the average biomass per plant against day of the year. The number of plants remains roughly the same during the reproductive period of the beetles. Therefore total biomass in the field is mainly influenced by the average biomass of the plants. Thus, although it is impossible to count the absolute number of larvae in the field, using these approximations, it is still possible to estimate the proportion of the total seasonal population of larvae that is present at a certain moment. The formula used to calculate the parasitism level of the flea beetle population over the whole season (PS, or Season-wide Parasitism) is:

$$\mathrm{PS} = \sum_{i=0}^{k} \left(\frac{p_i \cdot d_i \cdot b_i}{\sum_{i=0}^{k} (d_i \cdot b_i)} \right) \tag{1}$$

where p_i is the measured parasitism level at time i. This formula gives a weight to p_i. The effect of p_i on PS is largest when the largest proportion of the total seasonal population of the flea beetle larvae is present at moment i. This is when the product of d_i and b_i is largest.

Emerging parasitoids were stored in 70% ethanol, and identified to species level.

Results

Plant growth

The results of plant growth are shown in Fig. 3a (flowering data) and in Fig. 3b (biomass data). On average 120 plants for each *Sinapis* data point, and 246 plants for each *Barbarea*

point were measured. No *F*-value could be calculated for the trend line for flowering frequencies of *Sinapis*, because only three points were used to calculate the line. All other trend lines had a significant fit (*F*-test, $P < 0.05$). The calculated peak dates were 1 June for the flowering of *Barbarea*, 27 June for the flowering of *Sinapis*, 16 June for the biomass of *Barbarea* and 9 August for the biomass of *Sinapis*. Thus, averaged across the locations, *Barbarea* grew and developed at least 3 weeks earlier in the season than *Sinapis*.

Flea beetle reproduction and parasitism frequency

The average density of flea beetle larvae in Ejby (the only location where the exact number of collected leaves was counted) peaked earlier on *Barbarea* (15 June 2006) than on *Sinapis* (30 June 2006; Fig. 3c). After these respective dates, flea beetle densities decreased, and on July 16 the density of flea beetle larvae on both plant species had approached zero.

Two parasitoid species were reared from the collected samples: *D. morosus* and *A. brevicauda*, the latter only from larvae collected on *Sinapis*. Figure 3d shows the trend in the overall (irrespective of the parasitoid species) proportion of parasitized flea beetle larvae collected on *Sinapis* and *Barbarea* in Ejby. The maximum percentage of parasitism by *A. brevicauda* was 9.6% (*Sinapis*, 30 June 2006, n = 324), while the maximum percentage of parasitism by *D. morosus* was 83.3% (*Sinapis*, 7 July 2006, n = 72).

Early in the season of 2006, parasitism in Ejby was significantly higher on *Barbarea* than on *Sinapis* (Chi-square = 31.21, $df = 1$, $P < 0.001$ at the time with the largest difference, 15 June 2006, with 19.5% parasitism on *Barbarea* [n = 415] and only 6.2% on *Sinapis* [n = 387]; Fig. 3d). On 23 June 2006 parasitism on *Barbarea* was 10.2% (n = 176), while on *Sinapis* it was only 4.6% (n = 241) (Chi-square = 5.04, $df = 1$, $P < 0.025$). On the other dates no significant difference in parasitism between the two plant species was found.

The season-wide parasitism (PS) for larvae on *Barbarea*, calculated with formula 1, was estimated to be 19.1%, while the PS for larvae on *Sinapis* was 34.1%. Thus, the overall (season-wide) chance for a flea beetle larva to become parasitized is nearly twice as high on *Sinapis*, compared to *Barbarea*. The difference between the two plant species (15.0%) is caused by a 5.5% higher parasitism by *A. brevicauda,* and a 9.5% higher parasitism by *D. morosus* on *Sinapis*. The difference between the values of PS between larvae on *Sinapis* and on *Barbarea* is mainly caused by the results of the collections of 7 July, the last date that significant numbers of larvae where present and collected, when parasitation rates were high on both *Sinapis* and *Barbarea*, but larval densities had already ceased on *Sinapis*.

The (less detailed, because the collected leaves were not counted) results of the field observations done in Kværkeby largely correspond with those found for Ejby: parasitism in the late collection was significantly higher than in the early collection (*Barbarea*: $\chi^2 = 246.52$, $df = 1$, $P < 0.001$; *Sinapis*: $\chi^2 = 49.29$, $df = 1$, $P < 0.001$). The proportion of parasitism late in the season was very high (96% on *Barbarea* [n = 169], 97% on *Sinapis* [n = 77]). Parasitism on *Barbarea* in Kværkeby, however, was much lower (19%, n = 270) than parasitism on *Sinapis* (50%, n = 90) in the same area early in the season ($\chi^2 = 29.20$, $df = 1$, $P < 0.001$). Flea beetle larvae collected on *Barbarea* again were never parasitized by *A. brevicauda*, whereas larvae on *Sinapis* were, albeit with a very low percentage (2% in the early collection, n = 90).

Discussion

The results clearly show that, in our study locations, *Barbarea* grew and developed earlier in the season than *Sinapis*, and that flea beetle larvae peaked several weeks earlier on our studied *Barbarea* patch than on a nearby *Sinapis* patch. At the end of the season, when parasitism frequencies caused by *D. morosus* were extremely high, the population density of flea beetle larvae in Ejby on *Barbarea* was very low, while on *Sinapis* it was still relatively high. The population of larvae on *Barbarea* thus was able to escape from this high parasitism load. The population of larvae on *Sinapis* peaked around the time of the highest parasitism levels, and therefore in the end suffered from higher overall seasonal parasitism, whereas, paradoxically, on each individual sampling date in Ejby, the percentage parasitism on *Sinapis* was never higher (and on some dates actually significantly lower) than on *Barbarea* (Figs. 1, 3d). This clearly illustrates the importance of estimating host population size along with percentage parasitism; without this, in our study we would have drawn the opposite conclusion from the one we drew when including the host population size.

Also the fact that never a single *A. brevicauda* adult emerged from larvae collected on *Barbarea* contributed to the difference in enemy-induced mortality between the patches containing the two different plant species. Parasitism by *A. brevicauda* reached levels up to 15% on *Sinapis*. Concluding, we show that the patches with *Barbarea* indeed provided enemy-free space (or perhaps rather: enemy-free time) to larvae of *P. nemorum*, at least at the times and localities that we studied. The enemy-free space appears to be mainly caused by an asynchrony in phenology of the host and the parasitoids, as was also seen in the classical example of the apple maggot fly (Feder 1995).

Although the general pattern of parasitism across the different host plants was similar for Ejby and Kværkeby (which were the two localities at which both *Barbarea* and *Sinapis* were sampled), there was one marked difference: whereas in Ejby, parasitism on any individual collection event on *Barbarea* was never lower than on *Sinapis* throughout the season, in Kværkeby it was significantly lower on *Barbarea* than on *Sinapis* early in the season. This difference highlights the variation in parasitism that may be present across geographic locations. Within our study localities (Ejby and Kværkeby) the patches with the different host plant species were in close proximity. Therefore, we consider it unlikely that other factors than those associated with the presence of different host plant species are responsible for the observed differences in parasitism, especially since the general pattern was similar for the two different locations which were separated by approximately 45 km. Based on this, our conclusion is that it is the host plant *Barbarea* itself that provides enemy free space in our study areas. Of course the mere fact that *B. vulgaris* is available relatively early in the season may also influence the fitness of *P. nemorum* using it as host plant, irrespective of parasitism, by effectively advancing the possibility for reproduction. This, next to lower parasitism on *B. vulgaris* as was found in the present study, may provide an additional reason why *B. vulgaris* is used as host, despite its lower physiological suitability as food plant. Thus, although our study shows that *P. nemorum* using *B. vulgaris* escapes from a high season-wide parasitism-load, we can not conclude from these data that enemy-free space is the *primary* reason for the inclusion of *B. vulgaris* in the diet. In any case, our results unambiguously show that in further studies on the levels of parasitism in multi-trophic systems, sampling should (a) be season-wide, and (b) include the estimation of host population size at each sampling occasion, to estimate the overall season-wide proportion of the cohort of hosts that is parasitized, especially if phenological effects are suspected.

The fact that during this research *A. brevicauda* was only found on *Sinapis*, and not on *Barbarea*, indicates that *A. brevicauda* may have a behavioural preference for (host-infested) *Sinapis*. Many insect parasitoids have been shown to rely on a series of physical and chemical cues to find their hosts (Vinson 1976; Vet and Dicke 1992; Vos et al. 2001; Gols et al. 2005). Variation in these cues can be reflected in the attraction of parasitoids to certain plant species (Bukovinszky et al. 2005). This, in turn, would then create refuges for the host. Such refuges may be further influenced by host plant structure, host plant associated spatial aspects, etc. (Gols et al. 2005). For the flea beetle parasitoids studied here, no such studies of behavioural responses to odours have been performed until now. Whereas for *A. brevicauda* our present data support the existence of behavioural preference, this is not clear for *D. morosus*, which parasitized hosts on both plant species that were studied. The latter may have an interesting implication. We show that there is a certain proportion of flea beetle larvae living in *B. vulgaris* leaves early in the season that is parasitized by *D. morosus*. This may lead to an early start of the build-up of the *D. morosus* population. If these parasitoids can locally shift to other host plants, e.g. *Sinapis*, they might do so later in the season when flea beetle populations on *Sinapis* have started to build up. This, in turn, would provide an extra contribution to the extremely high parasitism of flea beetles on *Sinapis* late in the season. This can be considered a form of 'apparent competition' (albeit within one species) between flea beetles using the different host plants (Holt and Lawton 1993, 1994).

The apparent difference between the parasitism levels of *P. nemorum* on different plants by the different parasitoid species highlights the notion that refuges for organisms of lower trophic levels may be provided in various ways (Gols et al. 2005). *B. vulgaris* may represent a refuge for *P. nemorum* against *A. brevicauda* in various ways, including based on infochemical cues, plant structure, complexity of surrounding habitat and temporally. With respect to *D. morosus*, the temporal refuge provided by *B. vulgaris* appears the most likely explanation of its use as a host plant; *D. morosus* does parasitize *P. nemorum* on *B. vulgaris*, in some instances at even higher levels than on *S. arvensis*. This makes the possibility of *B. vulgaris* being a refuge in an infochemical or structural sense less likely. Resolving the mechanisms whereby host plants provide refuges to phytophagous insects is important, since such refuges can influence the communities and stability of ecosystems (Gols et al. 2005).

Concluding, when looking at each separate collection date, parasitism in one of the locations of our study was never lower on the alternative host than on the old host, whereas, considering the whole season, the selection pressure through parasitism wás lower on the alternative host than on the old host. This shows that information about parasitism levels at only a certain moment in time needs not be indicative of the total impact of parasitism on the entire cohort of that season. Therefore, when studying the role of enemy free space, it is in some cases, notably those where phenological differences across host plants are expected, essential that data about the number of hosts are collected simultaneously with data about parasitism level. Since within- and between season variation of the impact of parasitoids can generally be high in natural systems (Scheirs and De Bruyn 2002; Heard et al. 2006), it is essential that regular monitoring takes place over the entire season, and preferably even across multiple seasons (Scheirs and De Bruyn 2002).

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