

Food-choice of resistant *Phyllotreta nemorum* beetles between *Barbarea vulgaris* and *Raphanus sativus* plants

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Summary

Plants and herbivorous insects often influence each other's evolution. Interaction between the two has led to adaptations on both sides, which is called co-evolution. An example of co-evolution is the process of mutual adaptation between the flea beetle *Phyllotreta nemorum* and the cruciferous plant *Barbarea vulgaris*. Some populations of flea beetles show resistance to the defences of *B. vulgaris*. The common occurrence of resistant flea beetles on *B. vulgaris*, versus their rarity on other host plants, poses the question about the cause of this geographic distribution of resistance-phenotypes. One of the possible mechanisms is the existence of preference of different flea beetle genotypes for their respective host plants. The question of a possible preference of resistant *P. nemorum* flea beetles for *B. vulgaris* was investigated with experiments using a two-choice olfactometer and bioassays. The conclusions are: the flea beetles seem to smell damaged *Barbarea* and radish plants from a distance. Flea beetles have a preference for radish above *Barbarea* plants when they have to choose from a distance, but they appear to have a preference for *Barbarea* above radish, when they can touch the leaves.

Introduction

Plant-insect interactions are the product of and influenced by co-evolution between plants and insects. Co-evolution is the gradual change of hereditary characteristics in interacting species, as a reaction to reciprocal influences on each other. Co-evolution has led to adaptations of plants to the behaviour of plant eating insects, and vice versa. An example of adaptation is the defence of plants against insect damage. This occurs by physical structures, morphology and chemistry (Schoonhoven *et al.* 2005). The plant can defend itself either directly or indirectly. Examples of direct defences are toxins and thorns. By indirect defence the plant attracts for example carnivorous insects (Dicke & Vet, 1999) which subsequently attack the herbivores that threaten the plants. Shelters, alternative food, like nectar and production of chemical compounds are adaptations to attract carnivorous insects (Dicke & Vet, 1999). Chemicals can be used as an arrestant, repellent, stimulant and deterrent. Another example of adaptation is the resistance of plant eating insects to the defences of the plants. Plant defence generally leads to decreasing consumption of the plant. By natural selection however, some plant eating insects have developed into specialists, which can even use the plant defences as signal to recognise their food plant (Schoonhoven *et al.* 2005).

One of the aspects that have to be investigated to understand co-evolution of plants and plant-eating insects is the aspect of food choice or the food selection behaviour of the insects. The general mechanism of food selection behaviour is described in seven steps (Schoonhoven *et al.* 2005). The first step is no contact. Then, in the second step, the insect receives plant-derived cues that can be optical and/or olfactory. In the third step the insect responds to the cues; the distance between the plant and the insect decreases. When the plant is found, contact will follow by touching, climbing or landing on it. The surface will be examined by contact of the insect with the plant. In the next step the plant may be damaged and the content of the tissues are released by nibbling, test-biting, probing or puncturing with the ovipositor. During the last step either the plant will be accepted for egg laying, as food or the plant will be rejected (Schoonhoven *et al.* 2005).

Insects can be monophagous, oligophagous or polyphagous (Schoonhoven *et al.* 2005). Monophagous insects will eat from only one or a few closely related plant species. Oligophagous insects will feed on a number of plant species from the same plant family. Polyphagous insects can feed themselves with plants from different families. Polyphagous insects are called generalists (Schoonhoven *et al.* 2005). Mono- and oligophagous insects are called specialists. Specialists are rather the rule than the exception (Schoonhoven *et al.* 2005).

A case study of co-evolution is the process of mutual adaptation between the flea beetle *Phyllotreta nemorum* and the cruciferous plant *Barbarea vulgaris*. The flea beetle, *Phyllotreta nemorum* L. (Coleoptera: Chrysomelidae) (de Jong & Nielsen, 1999; Nielsen & de Jong, 2005), is an oligophagous species (de Jong *et al.*, 2001) using plants within the family of Brassicaceae (Nielsen & de Jong, 2005). Larvae are leafminers and adults feed on the same plant (de Jong *et al.*, 2001). The most commonly used host plant is *Sinapis arvensis* L. (Nielsen & de Jong, 2005). Other species of host plants are *Cardaria draba* (L.) Desv., cultivated radish (*Raphanus sativus* L.) and *Barbarea vulgaris* R. Br. (Nielsen & de Jong, 2005). Two types (G- and P- type) of *Barbarea vulgaris* ssp. *arcuata* exist. The two types differ in leaf pubescence and in resistance to the flea beetle. The G-type has glabrous leaves and is resistant to the flea beetle. The P-type has pubescent rosette leaves and is susceptible to the flea beetle (Agerbirk *et al.* 2003). In Denmark two varieties of *P. nemorum* exist of which one variety has been found on *Barbarea vulgaris* ssp. *arcuata* (G-type), using this plant as host (de Jong *et al.*, 2001). Normally this plant is unsuitable for the survival of the majority of the flea beetles (de Jong *et al.*, 2001) during spring and summer in the field (de Jong *et al.*, 2000). However, the beetles found on this plant have been shown to be resistant to its defences.

Genetic studies have shown that resistance to *Barbarea vulgaris* (G-type) has a genetic base in the form of a (or a few) major R-gene(s). These genes provide all stages of the beetles with the ability to feed on *Barbarea vulgaris*, whereas larvae lacking these R genes die when feeding on this plant (de Jong & Nielsen, 2002). The percentage resistant beetles on other plants than *B. vulgaris* is much smaller than found on *Barbarea* (Nielsen & de Jong, 2005). The question is why the percentage of resistant beetles on other plants is much smaller. One suggestion is that there is less migration of resistant beetles between plant species than one might expect, because of a preference of resistant individuals for *B. vulgaris* plants.

To examine the possible preference of flea beetles for *Barbarea vulgaris* plants the following research questions in the thesis are investigated:

“ Do resistant flea beetles (*Phyllotreta nemorum*) have a preference for *Barbarea vulgaris* and do olfactory cues have any influence on the host plant choice?”

“ Does the distance between flea beetle and plant influence the host plant choice?”

The distance is examined to investigate the possible difference in food- choice between different steps in the food selection behaviour.

Hypotheses

- Resistant flea beetles have a preference for *Barbarea vulgaris* compared to radish.
- Olfactory cues have influence on detection of the host plant of resistant flea beetles.
- The distance between flea beetle and plant (contact or no contact with the host plant) influences the host plant choice.

The research questions have been investigated with a two-choice olfactometer and bio-assays.

Material and Methods

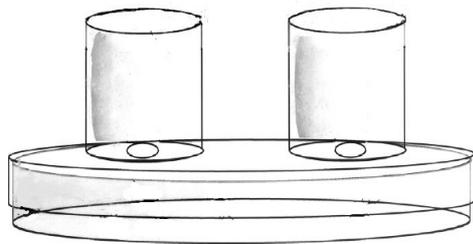
The following materials were used:

Flea beetles (*Phyllotreta nemorum* L.) which were resistant and susceptible to *Barbarea vulgaris* ssp. *arcuata* G-type. The beetles were kept in plastic vials (158 ml) (de Jong *et al.*, 2000) with a moist charcoal–gypsum bottom layer and closed with a plastic lid with a hole which is plugged with cotton wool. The beetles and larvae were reared on *Raphanus sativus* L. in a climate chamber with a temperature of 24 °C for the adult beetles and 22 °C for the larvae. There was a L18: D6 light-dark cycle in the climate chamber.

Leaves of the following plants: *Barbarea vulgaris* ssp. *arcuata* (G-type)
Raphanus sativus L. (radish)

The plants were reared in a climate chamber or greenhouse with a temperature between 24 °C and 36 °C and a L18: D6 light-dark cycle.

Olfactometer (van Tol *et al.*, 2002) which consists of: A Petri dish (140 mm diameter × 20 mm high), with two holes in the lid of 12mm in diameter with a distance of 95 mm from each other.



Above the holes are Plexiglass cylinders with a length of 65 mm and a diameter of 60 mm, in such a way that the hole is in the middle of each cylinder.

On top a lid was placed to avoid that the olfactory cues fleet.

Figure 1. Olfactometer

Both sexes of the *Phyllotreta nemorum* beetles were used. Of the newly emerged adult beetles, 98.6 % were known to be non-fed until the experiment, so they had no food experience. The other 1.4 % had access to radish leaves in the vial after emerging. The beetles were placed, one at the time, in the middle of the Petri dish of the olfactometer. Three to eight leaves of the test plants were placed in each of the cups. The surface of the leaves was of similar size. The beetles had the choice to go in the left or the right cylinder. Each beetle had 30 minutes time to choose. If they had not chosen after 30 minutes, the result was written down as no choice, with a remark at which side the beetle was at that time.

The following combinations were tested:

Barbarea G-type (damaged leaves) versus nothing

as control, to test if the set-up worked and

Barbarea G-type versus *Raphanus sativus* both with damaged leaves

Barbarea G-type versus *Raphanus sativus* both with undamaged leaves

All combinations were tested with resistant beetles to the defences of *Barbarea vulgaris* ssp. *arcuata* G-type. The leaves were always placed in the same cup. So there was no need to clean the cups between the experiments. The sides of the leaves in the cups were randomly chosen.

First I used the olfactometer with an extra cylinder on top of the first cylinder with gauze between the two cylinders. On the gauze the undamaged leaves were placed.

Bio-assays

I performed two kinds of bio-assays; one 2-choice bio-assay and one no choice. The 2-choice was to test if the flea beetles have a preference for *Barbarea* or radish, when they can touch the leaves. The no-choice was to determine the percentage of resistant beetles in the rearing (expected 100%). For the bio-assays I used plastic vials (158 ml) with a moist charcoal–gypsum bottom layer, closed with a plastic lid with a hole which is plugged with cotton wool. Punches of leaves of 1.4 cm in diameter were stuck to the bottom layer with pins about 1 to 1.5 cm from each other. For the 2-choice experiments there was one punch from *Barbarea* and one punch from radish. For no-choice two punches from *Barbarea* plant leaves were used. Each time some susceptible beetles were also used to check if the *Barbarea* plants were toxic for the beetles. One beetle was placed per vial; they were kept three days in a climate chamber with a temperature of 24 °C and a L18: D6 light-dark cycle. After three days I looked if the beetles had been eating or not and if there had been a preference. The preference was determined by visually estimating from which punch the beetle had eaten most.

Statistical tests

Binomial tests were carried out on the results of the olfactometer experiments and bio-assays.

Results

Olfactometer

<i>Barbarea</i> (damaged leaves) vs. no leaves	With the choice between damaged <i>Barbarea</i> leaves and no leaves the flea beetles chose significantly more for the <i>Barbarea</i> side (binomial $n = 78$, $p = 0.0009$)
<i>Barbarea</i> vs. radish (damaged leaves)	With the choice between damaged <i>Barbarea</i> leaves and damaged radish leaves the flea beetles chose significantly more for radish (binomial $n = 74$, $p = 0.0474$)
<i>Barbarea</i> vs. radish (undamaged leaves)	With the choice between undamaged <i>Barbarea</i> leaves and undamaged radish leaves the flea beetles did not choose significantly more frequently for either side (binomial $n = 77$, $p = 0.3620$)

Bio-assay

2-choice	The flea beetles ate significantly more from the <i>Barbarea</i> punches, than from the radish punches (binomial $n = 62$, $p < 0.0001$), as determined from the percentage of the leaf disk surfaces remaining.
no-choice	Of the 68 tested beetles, 97 % had eaten from the <i>Barbarea</i> punches. Those beetles are likely to be resistant to the defences of <i>Barbarea</i> .

Conclusions

- The flea beetles seem to smell damaged *Barbarea* and radish plants from 2.0 cm distance.
- Flea beetles have a preference for radish above *Barbarea* plants when they have to choose from a distance, i.e. step three of the food selection process.
- Flea beetles seem to have a preference for *Barbarea* above radish, when they can touch the leaves, i.e. step four to seven of the food selection process.

Discussion

Experiments

Olfactometer

With experiments with extra cylinders on top the flea beetles did not have a significant preference for either side, so the beetles did not seem to smell the leaves from that distance. Therefore I removed the top cylinders and used damaged leaves which I tore apart in 6 to 8 equal pieces.

During the experiment “*Barbarea* vs. nothing”, I discovered aphids on the plants. I have taken out the results of those repeats to check if the aphids had influence on the choice of the flea beetles. I performed a binomial test on the results of the flea beetles from the repeats with aphids on the *Barbarea*. The outcome of this test is that there is no significant difference in the choice of the beetles between *Barbarea* leaves and no leaves (binomial $n = 21$, $p = 1.0000$).

Bio-assay

Because the percentage of beetles that ate from *Barbarea* is so high, we can safely assume that all the beetles in the rearing were resistant.

With the bio-assays I looked at the percentage eaten from the leaf punches.

The amount of eaten punch, however, is difficult to estimate because radish punches (av. 55.65 mg) are relative heavier and ticker than *Barbarea* punches (av. 29.41 mg). The difference in weight can be explained by assuming that radish has more fluid in the leaves than *Barbarea* (Pers. Comm. P.W. de Jong). The percentage eaten punch has to be corrected for the weight per surface area of the different plant species. This has to be done because when looking only at the surface, the eaten volume is not always the same with different plant species. However no correction can be done on the percentage eaten punch because the radish punches become lighter (av. -3.27 mg) during the experiment and the *Barbarea* punches heavier (av. + 1.05 mg). An explanation could be that fluid evaporates from radish punches and that *Barbarea* absorbs fluid from the moist charcoal/gypsum bottom layer.

General

The result of the experiments confirm the hypothesis that olfactory cues have influence on detection of the host plant of resistant flea beetles. From a distance flea beetles have a preference for radish, whereas when they can touch the plants flea beetles have a preference for *Barbarea*. That is not consistent with the hypothesis that flea beetles have a preference for *Barbarea* above radish. However it is consistent with the hypothesis that the distance between flea beetle and plant influences the host plant choice.

New for investigation on the co-evolution of the flea beetle, *Phyllotreta nemorum* with the plant *Barbarea vulgaris*, is that from a distance resistant flea beetles have a preference for radish and when they can touch the plants flea beetles have a preference for *Barbarea*. As far as I know there are no other similar scientific researches with equal results.

In nature resistant flea beetles occur most on *Barbarea* plants and hardly on other plant species (Nielsen & de Jong, 2005). An explanation is a possible preference of resistant flea beetles for *Barbarea*. If both tested plants (the most used host-plant *Sinapis arvensis* was not tested) grow close to each other, resistant flea beetles, when they come from a distance, go initially to radish based on olfactory cues. This can be seen in the third step of the food-selection process. At step four and further the beetles are close to the plants and they go to radish or *Barbarea*. According to the preference for *Barbarea* in the bio-assays, the flea beetles move from radish to *Barbarea*, in the case that both plant species grow next to each other. Resistant flea beetles on other plant species compete with susceptible flea beetles. Finally there are more resistant flea beetles on *Barbarea* plants than on other plant species.

Some suggestions for further investigation about the preference of resistant flea beetles are;

- Experiments with *Sinapis arvensis* plants instead of radish plants, because this plant is the most commonly used host plant for the flea beetles.
- Experiments also with susceptible beetles to see if there is a difference in preference and/ or possible competition with resistant beetles.
- Experiments with more different distances, to examine were there is a change in host plant choice.
- Experiments with air flow olfactometer instead of still air olfactometer, because in nature there is also some air flow.

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