Olfactory response in caterpillars of *Pieris rapae* for host recognition

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Abstract

Plants interact with phytophagus insects through release of volatile chemical compounds. They can either function as a defense against herbivores by attracting predators and parasitoids, or they can act as an attractant for the herbivores themselves. This knowledge is important and could be applied in agricultural science to develop novel strategies for pest control. The plant-insect interaction was studied using three genotypes of the *Brassicaceae* family and the small cabbage butterfly, *Pieris rapae*. The experiment was conducted by placing larvae in a four-way olfactometer to see what potential feeding preferences it showed when choosing between the odor of 1) the three genotypes and 2) different treatments on each of the genotypes. The treatments tested were; undamaged plant, plants with larvae induced damage, frass and air as a control choice. One treatment experiment was made for each of the plant genotypes. The prediction was that larvae would show preferences for either i) damaged plants since the release of volatiles from these are more abundant that from undamaged plants, or ii) frass, since this emit nitriles which acts as an attractant for phytophagus insects. The result however showed no significant preference for either genotype or treatment, except for the treatment of cauliflower where the larvae seem to prefer the odor of damaged plants over that of the control choice. A number of volatiles are released exclusively or occur at higher concentration in the headspace of damaged cauliflower than in damaged plants of the other genotypes. This is what makes the cauliflower blend of volatiles unique and is most likely what attracts the larvae to it.
Introduction

Studies of plant – herbivore interactions are important for understanding the ecology of such mechanisms. Knowledge about this can be applied in agricultural sciences, for example, to develop new strategies for integrated pest management and is important if we are to reach a sustainable agricultural development.

The release of volatiles is a way for Brassicaceae plants to communicate with their surrounding environment (Ahjua et al. 2009). Different mixtures of volatiles are involved in different biological functions related to insects (Ahjua et al. 2009). Plants exposed to herbivory release volatiles originating from the glucosinolate-myrosinate defense which counteracts the insect attack (Miles et al. 2004). Brassicaceae is a family of cabbage species which has a chemical defense based on glucosinolates (a secondary plant metabolite) that is induced by tissue damage (Wittstock et al. 2004). When the tissue is damaged glucosinolates comes into contact with an enzyme, myrosinase (which in undamaged tissue is spatially separated from glucosinolates), and through hydrolysis of the glucosinolates, toxic and volatile products such as isothiocyanates are produced (Miles et al. 2004). These chemical compounds are considered to have a negative effect on and to be toxic to insects (Vergara et al. 2006). But there are herbivores like larvae of Pieris rapae which are specialized on feeding from plants that contain a high dosage of glucosinolates (Miles et al. 2004). These larvae are not affected by the toxic compounds from the deterioration of glucosinolates and they even use them as feeding stimuli (Miles et al. 2004). They are able to do this thanks to a protein (nitrile specifier protein, NSP) in the larval gut which prevents isothiocyanates from forming and instead makes the product of the hydrolysis to be nitriles (which are far less toxic) (Wittstock et al. 2004). The nitriles are then excreted in the larval frass (Hopkins et al. 2009). In plants such as those belonging to the Brassicaceae family, glucosinolates stimulate specialist species to recognize the plant as a host and at the same time repels generalist phytophagus insects (Hopkins et al. 2009).

Plants can also release volatiles that act as an attractant for predators and parasitoids on the attacking herbivore, thus serving as an indirect defense (Ahjua et al. 2009). Glucosinolate-derived volatiles (isothiocyanates, oxazolidine, thiocyanates, epithionitriles, thiones and nitriles) also act as attractants for some herbivore insects (Ahjua et al. 2009), they trigger ovipositioning in many Lepidopteran species and they stimulate feeding in larvae (Hopkins et al. 2009). This suggests that the mustard-oil bomb (Rohloff et al. 2005) defense has two functions; i) its defensive function against herbivory already described and ii) the function of an attractant of specialist phytophagus insects. Cabbage butterfly (P. rapae), being a specialist on Brassicaceae species (Ahjua et al. 2009), should therefore show preference for plants which release a high amount of volatiles.

Ovipositioning adults choose food for its offspring when they decide what plants to lay their eggs on (Chew 1980). Why then do the larvae need to be able to find and distinguish between foodplants themselves? It is known that ovipositioning adults may lay eggs on plants which does not support all of the larval development stages (they might be too small or of the wrong species) and many larvae which are placed on good foodplants still needs to move to another plant to find food in later instars (Chew 1980). This implies that the ability to find food other than that chosen by the ovipositioning adult is needed by the larvae and it should therefore be able to show preferences in food selection.

In this study I examined what potential feeding preferences larvae of P. rapae have when choosing between i) three genotypes of Brassica oleracea L. and ii) different treatments of plants from each of
the three genotypes. The three treatments used where; an undamaged plant, a plant with larvae induced damage and a petridish containing frass. Plant tissue that has been damaged by chewing insects’ exudate volatile attractants (Miles et al. 2004); therefore should preferences be shown by larvae for the damaged plants since these plants would exudate more of these compounds than the undamaged ones. However, the thought of nitriles also being a stimulant for feeding and host recognition arises. Since larvae excrete nitriles in their frass, nitriles might indicate the presence of feeding conspecifics and thereof the species host plant. Therefore should frass perhaps be the treatment preferred by the larvae in this experiment. In a study by Mumm et al. 2008 they tested if caterpillars of *P. rapae* preferred feeding on plants that mainly produced isothiocyanates or nitriles. The larvae fed just as much on both and showed no preference. This gives support for the prediction of frass being an attractant since it contains nitriles. Something that contradicts this is that nitriles might signal the presence of conspecifics and a higher intra/interspecific competition and lack of food (Mumm et al. 2008). It might also be that the larvae express avoidance behavior for frass since nitriles are known to attract parasitoids such as *Cotesia rubecula*, which is a specialist parasitoid on larvae of *P. rapae* (Mumm et al. 2008).

*Species biology*

The larvae are green with yellow stripes running along its back. During its larval development it has five instars and size varies between 3.2 – 30.1 mm depending on what instar it is in. It requires 11 -33 days to complete its development depending on the temperature. When the larva reaches its fifth instar it pupates and then after approximately 11 days a butterfly emerges. The butterfly is about 4.5 - 6.5 cm between the tips of its wings. They can also go into diapause and use this as a way of overwintering. In that case they can stay as a pupa for several months (Capinera 2008, Ross et al. 1998). The larvae are specialists on *Brassicaceae* plants (Ahuja et al. 2009) and utilize these as hosts and can cause a lot of damage to agricultural crops, thus being an economically important pest species (Mozuraitis et al. 2011).

One key for both caterpillars and adults to find their host plants is olfactory cues (Bruce et al. 2006). The adults have olfactory receptor neurons, located primarily on their antennas, which interpret chemical signals and send information about this to the insect central nervous system (Bruce et al. 2006). This allows the butterfly to distinguish between host and non-host plants from a distance, even though ovipositioning is not triggered until exposure of other chemicals (for example isothiocyanates) upon landing (Mumm et al. 2008).

Not much has been written on olfactory reception of host plant volatiles in the caterpillars. Chew 1980. writes that “larvae show limited ability to distinguish between crucifers (*Brassicaceae* plants) and non-crucifers” and that “larvae wandering a few millimeters away from crucifers sometimes missed them”. This indicates a poor ability for olfactory reception in caterpillars of *P. rapae*, but then he also writes that larvae from the latest instar (the 5th) often turns towards crucifers from longer distances ( up to several centimeters), though it is not clear if they do so due to olfactory stimulation or the reception of moisture gradients.

*Hypotheses*

The aim of this study was to test if larvae of the specialist insect *Pieris rapae* are able to distinguish between three *Brassicaceae* genotypes (cabbage, cauliflower, broccoli) and three plant treatments.
According to the results from a headspace analysis of volatiles released by cabbage, cauliflower and broccoli, the release of volatile compounds is most abundant from cabbage (Mozuuraitis et al. 2011); pursuant to this the hypothesis that cabbage would be the preferred genotype was formed. As a defensive function, plants that have been damaged by chewing insects release more volatiles than undamaged ones. This change in the release of volatiles (quantitative or qualitative) attracts phytophagus insects. A hypothesis was formed which predicted that damaged plants would be preferred by the larvae since this indicate the presence of a suitable feeding substrate. Frass contain nitriles which also attract herbivore insects; this led to the hypothesis that frass would be the preferred treatment.

Material and Methods

The plants:

The plants used in the experiments were grown from seeds in a greenhouse at the ecology department at SLU, Uppsala. After they had reached a height of approximately 20-30 cm they were used in the experiments. The three different genotypes of the cabbage species Brassica oleracea L. that were used were; cabbage (B. oleracea var. capitata), cauliflower (B. oleracea var. botrytis), broccoli (B. oleracea var. cymosa).

The larvae:

The species that was used were caterpillars of the cabbage butterfly Pieris rapae. To avoid inducing food preferences they were bred on randomly chosen plants of Brassica oleracea L inside of cages in a climate room at the ecology department at SLU, Uppsala. Larvae of the 2nd-4th instar were used in the tests and each one was only used once to avoid pseudo replicates.

The treatments:

Plants of each of the three genotypes were placed in a cage containing larvae (4th-5th instar) to generate plants with larvae induced damage needed for the experiment. The plants were left there for approximately 24 hours prior to the experiments. They were then cleaned from any frass residue. Frass were also collected from the same for further use in the experiments.

Genotype preference test:

The preference analysis was conducted using a four-way olfactometer system. The olfactometer is a container with four “arms” in which the larvae were placed (fig 1). Through a series of tubes the olfactometer is connected to four chimneys (fig 2) containing plants of the different genotypes. Air is sucked in from the four chimneys, through the tubes and then exits through another tube connected to the center of the olfactometer. Air flows from the chimneys, and in to the olfactometer through the four arms. The air carries the smell of the genotype connected to that arm and thus the larvae are exposed to the odor of each of the genotypes and are supposed to choose the most preferred one. The larvae are then presumed to move to and spend most of its time in the arm which its preferred foodplant is connected to. During the experiment four olfactometers were run at the same
time and the position of the tubes connecting the chimneys to the olfactometer were alternated between the arms of the olfactometer to avoid positioning effects. Light intensity was constant in all olfactometers so no arm would be preferred by the larvae due to light condition.

The air intake was set to 0.7L/min and the four different choices for the larvae to take were; undamaged broccoli, undamaged cauliflower, undamaged cabbage and air as a control choice. First the larvae was placed in the center of the olfactometer and then left there for 12 minutes to get acclimatized, after which its position in the olfactometer was recorded every 3rd minute until 10 positions had been registered. The olfactometer was then cleaned using 70% alcohol to be used in the next trial. In total, 18 replicates of the genotype preference test were made.

**Treatment preference test:**

For this test the same olfactometer system were used (the four-way olfactometer and chimneys). Now the larvae had the choice between; undamaged plant, damaged plant, frass and again air as a control choice. Why then should these treatments have any effect on the larvae behavior? The undamaged plants (cabbage, cauliflower and broccoli) releases a certain amount of volatile compounds depending on what species it is (Mozuraitis et al. 2011) and the larvae are presumed to react to these compounds when exposed to them. The plants with larvae induced damage also release volatile compounds but they are more abundant and which compounds that are released may vary between plant species (Mozuraitis et al. 2011). Frass is the feces of the larvae, this contain lots of nitriles (Hopkis et al. 2009) due to chemical reactions induced by the feeding larvae to topple the glucosinolate-myrosinase defense in plants. These chemicals attract herbivorous insects (Ahuja et al. 2009) and supposedly also attract the larvae of *P. rapae*.

*Cabbage (B. oleracea var. capitata)*

Cabbage plants with each of the treatments were placed in chimneys and these were connected to the olfactometer. Four olfactometer-systems were run at the same time so four of each of the treatments were used (one for each of the olfactometers). Position of the treatments (i.e. which arm
the chimney were connected to) was altered between sessions. The larvae were put in the center of the olfactometer and were left alone for 12 minutes to get acclimatized. Entries of the larvae position were made every 3rd minute. In total, 22 replicates were made.

*Cauliflower (B. oleracea var. botrytis)*

This experiment was conducted the same way as with cabbage. Four olfactometers were run at the same time and that required four samples of each of the different treatments. Positions of the treatments were altered between arms. In total, 20 replicates were made.

*Broccoli (B. oleracea var. cymosa)*

This experiment using broccoli as test plant was conducted two times. The first was conducted the same way as with cabbage and cauliflower and 22 replicates were made. The second one was conducted to ensure that there was no positioning effects (i.e. that the larva position at one time was not dependent of the previous/later one), therefore the test was repeated but with five minutes between entries, thus giving the larvae more time to move around. There was no specific reason why broccoli was chosen for the 5 minute interval test, the plant to perform this test on was chosen randomly among the genotypes. In total, 20 replicates were made.

The analysis:

The data obtained from the experiments was analyzed using Friedman’s ANOVA with a significance level of $p < 0.05$. Wilcoxon’s test for matched pairs was then used to analyze pairs of genotypes and treatments within the sample to see which one was the most preferred. This was also done at a significance level of $p < 0.05$.

**Results**

The genotype preference experiment:

The test was made to investigate if there was any significant difference in preference for any of the three genotypes. However I did not find any significant differences $p = 0.14$ (*fig.* 3).

The Treatment preference experiment:

*Cabbage (B. oleracea var. capitata)*

This test was conducted to examine if there was any significant difference in preference for any of the treatments on cabbage. The experiment did not generate any significant results that indicate preference for any of the treatments on cabbage $p = 0.33$ (*fig* 4).

*Cauliflower (B. oleracea var. botrytis)*

This test was conducted to see if there was any significant difference in preference for any of the treatments on cauliflower. The results reveal that there is a significant difference in preference between these treatments $p = 0.046$ (*fig* 5). When testing the data from within the cauliflower sample with Wilcoxon’s matched pairs test, it is revealed that damaged plants are preferred over the
control $p = 0.0097$. Also, there was a strong indication of both undamaged plants and frass being preferred over the control $p = 0.055$ and $p = 0.052$ respectively (table 1).

**Broccoli (B. oleracea var. cymosa)**

This test was conducted to see if there was any significant difference in preference for any of the treatments on broccoli. The results for both parts of the test (3 and 5 minute intervals) show no significant difference in preference for any of the treatment on broccoli $p_{3\text{min}} = 0.45$ and $p_{5\text{min}} = 0.38$ (fig 6 and 7).

![Boxplot of data from the genotype experiment](image)

**Fig 3.** Boxplot of data from the genotype experiment. The x-axis is the number of visits and the y-axis is the different genotypes. The graph shows number of visits in the arm corresponding to each of the different genotypes. There is no significant difference in number of visits (i.e. preference) for any of the genotypes.
Fig 4. Boxplot of data from the treatment experiment using cabbage as test plant. The x-axis is the number of visits and the y-axis is the different treatments of cabbage. The graph shows number of visits in the arm corresponding to each of the different treatments. There is no significant difference in number of visits (i.e. preference) for any of the treatments.

V = Undamaged cabbage  
Vs = Damaged cabbage  
F = Frass  
C = Control

Fig 5. Boxplot of data from the treatment experiment using cauliflower as test plant. The x-axis is the number of visits and the y-axis is the different treatments of cauliflower. The graph shows number of visits in the arm corresponding to each of the different treatments. There is a significant difference in number of visits (i.e. preference) for the treatments.

BL = Undamaged cauliflower  
BLs = Damaged cauliflower  
F = Frass  
C = Control
Table 1. Data from Wilcoxon’s matched pair analysis of cauliflower. Comparison between number of visits in each of the genotypes reveal a preference for damaged cauliflower over the control choice and a strong indication of preference for both undamaged cauliflower and frass over the control choice.

Wilcoxon Matched Pairs Test (BL BLd F C)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>p – value</th>
<th>Z</th>
<th>No. replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undamaged &amp; damaged cauliflower</td>
<td>0.54</td>
<td>0.615</td>
<td>20</td>
</tr>
<tr>
<td>Undamaged cauliflower &amp; frass</td>
<td>0.98</td>
<td>0.023</td>
<td>20</td>
</tr>
<tr>
<td>Undamaged cauliflower &amp; control</td>
<td>0.055</td>
<td>0.916</td>
<td>20</td>
</tr>
<tr>
<td>Damaged cauliflower &amp; frass</td>
<td>0.43</td>
<td>0.784</td>
<td>20</td>
</tr>
<tr>
<td>Damaged cauliflower &amp; control</td>
<td>0.0097</td>
<td>2,585</td>
<td>20</td>
</tr>
<tr>
<td>Frass &amp; control</td>
<td>0.052</td>
<td>1,939</td>
<td>20</td>
</tr>
</tbody>
</table>

Fig 6. Boxplot of data from the treatment experiment using broccoli as test plant and three minutes interval between entries. The x-axis is the number of visits and the y-axis is the different treatments of broccoli. The graph shows number of visits in the arm corresponding to each of the different treatments. There is no significant difference in number of visits (i.e. preference) for any of the treatments.
Discussion and Conclusions

The genotype preference experiment:

This experiment did not yield results that indicated any significant differences between preferences of any of the three genotypes; the larvae did not prefer the odor of any of them. Nevertheless Mozuraitis et al. 2011 recorded that 20, 24 and 19 volatile compounds where emitted from undamaged cabbage, cauliflower and broccoli. Cabbage was the one that had the most distinct mixture of volatiles meanwhile the ones from broccoli and cauliflower was more difficult to distinguish between. Pursuant to this I expected that the larvae in my experiment would show a significant preference for cabbage, though this was not the case.

It might be that the blends of volatiles in the three genotypes are so alike that the difference between them is too small for the larvae to detect and therefore they are not able to show preference for either of them. Another thing that might have interfered with the test was the time during which the larvae were deprived of food. If the larvae are too starved they would not reject an acceptable source of food over a preferred one (Chew 1980). Since it’s known that all three genotypes are acceptable food sources to P. rapae, this suggests that the larvae in this experiment...
might have been too hungry to distinguish between the most preferred genotype and the acceptable ones.

The Treatment preference experiment:

The larvae did not show any significant preference for any of the treatments (undamaged/damaged plant, frass, and the control choice which was air) on cabbage or broccoli; however in the test using cauliflower they showed a significant difference between the control (neutral odor) and the damaged plant. In the same study as mentioned earlier by Mozuraitis et al. 2011, it was recorded that 46, 28 and 26 volatile compounds where emitted from cabbage, cauliflower and broccoli that had been damaged by larvae of *P. rapae* (the difference in number of volatiles released between undamaged plants and plants in larv induced damage was 26, 4, and 7 respectively). This denotes that the most substantial difference (in the quantity of volatiles released) between undamaged plants and plants with larv induced damage was found in cabbage, followed by broccoli and cauliflower. Granted this I expected the larvae in my experiment to show preference for the plants with larv induced damage, at least in the test using cabbage. Since this was not the case, it suggests that there is a specific volatile compound or combination of compounds that is exclusively present in cauliflower but is absent in the other genotypes which attracts the larvae. Mozuraitis et al. 2011 tells that cauliflower damaged by larvae of *P. rapae* release a number of volatiles which are unique to that genotype or occur at a larger extent in it than the others ((E)-β-faranesene and approximately 10 different esters). This suggests that these compounds contribute to making the blend of volatiles unique and attractive to larvae. Results from this study coincide with the ones acquired in a similar one by Malmgren 2010. The experimental design was the same as in this study but she tested the preferences of caterpillars of *P. brassicae*. They also showed preference for damaged cauliflower plants but no significant preferences for any of the other treatments. This gives further support for the suggestion that the volatiles or blend of volatiles, released by cauliflower is exclusive to that genotype and that it is what attracts the larvae. However, it is not known exactly what chemical compounds, mixture of compounds or concentrations of them that attract the larvae. The release of volatiles may vary depending on the extent of larval infestation and at which stage in the plants development the attack occur. Older plants may be equipped with a better and more functional defense against herbivory than young ones, thus specialist larv which can overcome this defense may be more attracted to grown plants with larv induced damage since these plants may emit more volatiles. The timing and extent of the infestation could influence which chemicals are released and at which concentrations, thus changing the odor profile of cauliflower and, perhaps, making it more or less attractive to herbivores. It may also be that the cauliflower responds to herbivory in a different way than the other genotypes, the physiological respond induced in the plant when it’s exposed to herbivory may not be the same and this may also change the odor profile to be more or less attractive.

The larvae also showed a strong trend of preference for undamaged plants over the control (p = 0.055) and for frass over the control (p = 0.052) in the test using cauliflower. This could also be expected since, as well as damaged plants, undamaged plants release volatiles (Geervliet et al. 1997), and frass contain nitriles (Hopkis et al. 2009) which acts as an attractant for phytophagus insects (Ahuja et al. 2009).
To investigate if the non-significant results from the broccoli and cabbage experiments were due to positioning effects caused by the narrow time span in which the larvae had to change position in the olfactometer, the time between entries was increased. However despite the increase in time between entries from three to five minutes, there was no change in the results. Although the larvae was given more time to move around, thus ensuring the avoidance of positioning effects, the result still showed no preference for either treatment.

It’s been shown in several studies (Mumm et al. 2008, Ahuja et al. 2009, Shiojiri et al. 2010. Chew 1980. Geerlivet et al. 1997) that many insects respond in different ways to volatile compounds released from plants. Both specialist and generalist phytophagus insects use plant volatiles to locate their host plants, and predators and parasitoids use them to distinguish between infested and non-infested plants (Paré et al. 1999). These multitrophic plant-insect interactions are of great interest, not only from an ecological, but also from an economical point of view. Insect species that are considered pests can cause lots damage to crop and costs a lot of money. If we can learn more about how plants and insects interact, that knowledge can be put in to use within agriculture to prevent these problems. For example; development of trap crops to lure pest-insects away from the economically important crops (Ahuja et al. 2009), or instead of old broad range pesticides which are both harmful and costly to use we can treat plants with already naturally occurring chemical or semiochemical compounds (Ahuja et al. 2009). These will either deter phytophagus insects or attract predators and parasitoids of the herbivore insect. To be able to develop a sustainable agriculture without causing substantial damage to the environment, we need this kind of alternative pest control.

As for future directions it would perhaps be interesting to repeat the experiment, but with larvae reared on just one plant species to see if those larvae would show induced feeding preferences for the specific genotype it was reared upon. In the study by Mozuraitis et al. 2011, the one species of plants which had the most unique blend of volatiles were curly kale. It would be interesting to see the genotype experiment repeated using curly kale as one of the plant species to investigate if the larvae prefer it over the other genotypes.

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