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The effect of phenidone, an inhibitor of the octadecanoid pathway, on the oviposition preference of two cabbage white butterfly species.

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Summary

Plants have evolved several defence mechanisms against attack by herbivorous insects. The most important pathway involved in induced plant defence against herbivore attack is the octadecanoid pathway, inducing the production of defence compounds in plants upon feeding damage and oviposition by insects. The larvae of *Pieris rapae* and *P. brassicae* are specialist herbivores of *Brassica* plants. Plant defence compounds are induced by feeding larvae via the octadecanoid pathway, which will have a negative influence on the performance of the larvae. Furthermore, females prefer to oviposit on plants that are not yet infested with caterpillars.

Lipoxygenase, which is a key enzyme in the octadecanoid pathway, can be blocked by the redox-active compound phenidone. In this research, the effect of the inhibition of lipoxygenase by phenidone on the oviposition behaviour of *P. rapae* and *P. brassicae* was investigated. Furthermore, it was investigated whether both *Pieris* species discriminate between infested and uninfested Brussels sprouts plants.

P. rapae does not discriminate between infested and uninfested plants, in contrast to *P. brassicae*. Because of this, it could not be concluded whether treatment with phenidone did have an effect on the oviposition preference of *P. rapae*. Treatment with phenidone only had a weak positive influence on the oviposition of *P. brassicae*. Further research is needed to investigate whether phenidone treatment did not have an effect on the oviposition preference of *P. brassicae* and whether it will have an effect on the oviposition preference of *P. rapae*.

Introduction

About fifty percent of all insects rely on plants for food. Plants, having to cope with being attacked by herbivorous insects, have therefore evolved several defence mechanisms (Schoonhoven *et al*. 2005). Besides morphological defence mechanisms like spines or wax layers, chemical defence is extremely important. Chemical defence mechanisms can be constitutive, that is, the defence compounds are already present in the plant, independent of wounding caused by herbivores, or induced, which means the defence compounds will only be produced in response to herbivore attack. Defence compounds can have a direct influence on the herbivore, like toxic or repellent compounds, or have an indirect influence by producing compounds that attract natural enemies of the herbivore.

Pieris rapae (L.) and *P. brassicae* (L.)

The larvae of two cabbage white butterflies, *Pieris rapae* L. (Lepidoptera: Pieridae) and *Pieris brassicae* L. (Lepidoptera: Pieridae) are specialist herbivores of *Brassica* plants, including the economically important crop cabbage (*Brassica oleracea*). A single *P. rapae* caterpillar can eat an average of 27 cm² leaf area during its total development and a single *P. brassicae* caterpillar 76 cm² (Theunissen *et al*. 1985). Large groups of these caterpillars can thus cause a lot of damage to these crops.

The small cabbage white butterfly (*P. rapae* L.) lays her eggs singly and preferably on plants that stand alone (Davies and Gilbert 1985). The cryptically coloured larvae complete their larval development on a single host plant (Fig. 1). The large cabbage white butterfly (*P. brassicae* L.), on the other hand, lays her eggs in batches of 10 to 150 eggs and prefers clumped vegetation (Davies and Gilbert 1985, Le Masurier 1994). The aposomatically coloured larvae feed gregariously and migrate to another host plant once the original host plant is defoliated (Fig. 1). Furthermore, *P. rapae* females prefer to spread their eggs over different host plants, while *P. brassicae* females spend more time laying eggs on the same host plant (Stamp 1980).

Because newly hatched larvae have limited mobility, host selection by the mobile adults is extremely important to determine the offspring's fitness (Tabashnik 1987). In order to find a suitable host plant, butterflies make use of visual, olfactory and contact stimuli. Renwick and Radke (1988) found that visual and contact stimuli are more important cues in host plant selection by *P. rapae* butterflies than olfactory stimuli. In other words, it might be that they

are not as much attracted by plant volatiles as by optical cues, but this is not yet confirmed by further research.

As soon as the female *Pieris* butterfly has landed on a potential host plant, she relies on chemoreception to decide whether or not to accept the plant as oviposition site. As host plants may contain oviposition stimulants and non-host plants contain oviposition deterrents (Tabashnik 1987), the final acceptance of a host plant relies on a balance between different concentrations of oviposition stimulants and –deterrents (Huang and Renwick 1993, Renwick and Chew 1994). Important oviposition stimulants for *Pieris* butterflies are glucosinolates, naturally occurring compounds in all *Brassica* plants (Renwick and Chew 1994). Specific glucosinolate profiles may affect the attractiveness of plants as oviposition sites for *Pieris* butterflies. The glucosinolates glucobrassisin and sinigrin are found to be the most effective oviposition stimulants (Renwick *et al*. 1992, Van Loon *et al*. 1992).

However, *P. rapae* and *P. brassicae* avoid laying eggs on host plants that are already infested with herbivores (Sato *et al*. 1999, Shiojiri *et al*. 2002). This for several reasons, first, the eggs laid by the butterflies could be eaten by the already present herbivores and second, the newly hatched larvae would face more competition for resources (Shiojiri *et al*. 2002). Furthermore, herbivore attack induces the production of defence compounds in plants, which could have a negative effect on the performance of the just hatched larvae or attract their natural enemies (Shiojiri *et al*. 2002). *Pieris rapae* and *P. brassicae* females are known to switch to less acceptable host plants if eggs or larvae are already present on the preferred host (Stamp 1980).

Figure 1: Above: Eggs (left) and a caterpillar (right) of *P. rapae.* Source: http://www.unil.ch and www.pbase.com. Below: Eggs (left) and caterpillars (right) of *P. brassicae*. Source: http://www.museums.org.za

The octadecanoid pathway

There are three main pathways involved in induced plant defence, namely the octadecanoid pathway, the salicylic acid pathway and the ethylene pathway (Schoonhoven *et al*. 2005). These pathways do not always act apart from each other, but can also act synergistically or antagonistically (Pieterse *et al*. 2001, Schoonhoven *et al*. 2005). For example, both the octadecanoid pathway and the ethylene pathway play a role in the induced defence of Lima bean plants caused by spider-mite attack (Horiuchi *et al*. 2001), while these two pathways act antagonistically in case of nicotine production in wild tobacco (Kahl *et al*. 2000).

The most important pathway involved in induced plant defence against herbivorous insects seems to be the octadecanoid pathway (Dicke and Van Poecke 2002). This pathway is known to induce the production of defence compounds in response to oviposition as well as feeding damage by insects. Jasmonic acid (JA), a product of the octadecanoid pathway, is a major signal molecule in plant defence, as it induces the expression of genes leading to the production of several defence compounds (Dicke and Van Poecke 2002, Kessler and Baldwin 2002). The plant defence system can be induced by treating plants with JA. Bruinsma *et* al. (2007) observed that *Pieris* butterflies preferred control plants over JA-treated plants as oviposition sites. This was due to processes in the plant after JA treatment, as no difference in the number of eggs was observed between green cardboard paper treated with an oviposition stimulant and JA or green cardboard paper treated with an oviposition stimulant only. However, no changes were found in the leaf surface glucosinolate profile that could explain the observed behaviour.

Lipoxygenase (LOX) is a key enzyme in the synthesis of jasmonic acid induced by wounding via the octadecanoid pathway (Arimura *et al*. 2005). LOX transforms linolenic acid into 9- and 13-hydroperoxides, which are then converted to aldehydes and oxoacids (Kessler and Baldwin 2002). Products from 13-(S)-hydroperoxy linolenic acid can be further transformed by several enzymes to eventually produce JA (Fig. 3) (Kessler and Baldwin 2002, Koch *et al*. 1999). Kessler *et* al. (2004) observed that LOX-deficient plants are unable to produce defence compounds and thus are more susceptible to herbivore attack. Furthermore, herbivores gained weight faster when feeding on LOX-deficient plants than when feeding on plants with intact expression of JA. Octadecanoids derived from LOX activity might play a direct role in host plant selection by making it possible for herbivores to differentiate between plants with and without intact JA-signalling.

Phenidone, an inhibitor of the octadecanoid pathway

The redox-active compound phenidone (1-phenyl-pyrazolidinone) (Fig. 2) is known to inhibit LOX activity (Fig. 3) (Engelberth *et al*. 2001, Koch *et al*. 1999), by reducing the active form of LOX to an inactive form. This blocking of LOX activity makes phenidone an effective inhibitor of the octadecanoid pathway, and thus of the plant's induced defence system (Paré *et al*. 1996).

Indeed, Engelberth *et al*. (2001) found that Lima bean plants (*Phaseolus lunatus*) treated with 1mM phenidone did not emit any volatiles induced by the octadecanoid pathway. However, Kim *et al*. (2003) observed that LOX activity was not completely inhibited by treatment of plants with 1mM phenidone, but wound-induced expression of LOX was delayed from 3 to 6 hours. The lack of complete inhibition of LOX could be due to the fact that the inhibitory effect of phenidone is not specific to the biosynthesis of JA, as it also inhibits LOX from animal origin (Hlasta *et al*. 1991, Cucurou *et al*. 1991). In an experiment performed by Heil *et al*. (2001), phenidone was found to inhibit the flow of extrafloral nectar induced by the octadecanoid pathway in the plant *Macaranga tanarius* and Bruinsma (pers. comm.a) found that herbivore infested Brussels sprouts plants treated with phenidone were less attractive to the parasitoid wasp *Cotesia glomerata* than infested plants treated with a control solution. However, they were still more attractive then uninfested plants.

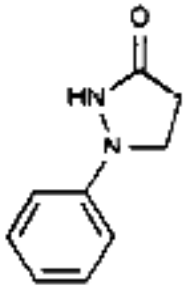

Figure 2: Structure formula of phenidone.
Source: Hlasta *et al*. 1999.

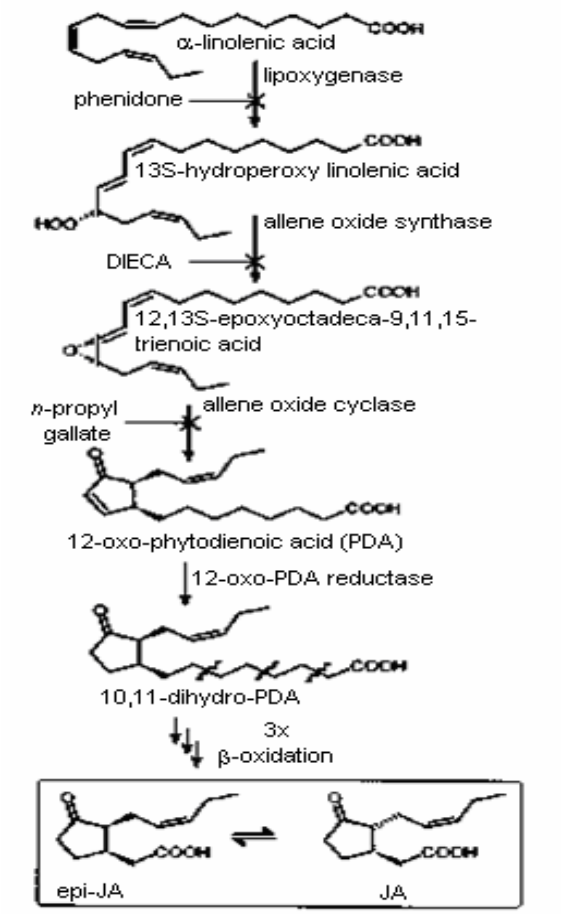

Figure 3: Representation of the pathway of jasmonic acid biosynthesis from α-linolenic acid. Different inhibitors are indicated. Source: Koch *et al*. 1999.

By inhibiting certain steps in the octadecanoid pathway, the importance of that particular step can be investigated. This research will investigate the effect of phenidone, which inhibits a step early in the octadecanoid pathway, on the oviposition behaviour of *P. rapae* and *P. brassicae*. It is hypothesised that treatment of plants with an inhibitor of the plant's induced defence system will positively influence the oviposition preference of the butterflies. The following questions will be addressed: 1) Do *P. rapae* and *P. brassicae* discriminate between herbivore-infested plants treated with phenidone and herbivore-infested control plants when choosing an oviposition site? 2) Do *P. rapae* and *P. brassicae* discriminate between plants that are infested with caterpillars and uninfested plants when choosing an oviposition site? 3) Is lipoxygenase-activity crucial in the octadecanoid pathway-mediated plant defence against herbivores? 4) Does phenidone treatment of plants affect the performance of *P. rapae* caterpillars? and 5) Is the observed effect due to phenidone itself or due to chemical changes in the leaf surface caused by phenidone? This will lead to increased knowledge about the interaction between herbivores and their host plants and the role of induced plant defence.

Materials and Methods[1]

Plants and Insects.

Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* L. (Brassicacea) cultivar Cyrus were grown in plastic pots of 11 x 11 x 11 cm in a greenhouse at 20-28°C, 40-80% RH and a 16L:8D photoperiod. All experiments were done using 6-7 weeks old plants.

Stock colonies of the small cabbage white butterfly (*Pieris rapae* L. Lepidoptera: Pieridae) and the large cabbage white butterfly (*Pieris brassicae* L. Lepidoptera: Pieridae) were maintained on Brussels sprouts plants in a climatised room at 20-22°C, 50-70% RH and a 16L:8D photoperiod.

Effect of pure phenidone on the oviposition preference.

To test the effect of pure phenidone on the oviposition preference of *Pieris rapae* butterflies, green cardboard paper of 8 x 11.5 cm was sprayed with 1 mL 5 mM sinigrin solution (Janssen Pharmaceutica, Tilburg, The Netherlands) using a Desaga chromatographic sprayer (Heidelberg, Germany). Sinigrin is a naturally occurring compound in Brassicaceae and known to be an oviposition stimulant for *Pieris* butterflies (Renwick and Chew 1994, Van Loon *et al*. 1992). The paper was allowed to dry and was subsequently sprayed with 1 mL 2mM phenidone solution or 1 mL water as a control, just before the paper was introduced into the cages with butterflies.

Pieris rapae butterflies seemed to have some trouble landing on cardboard paper. Therefore, in case of *P. brassicae*, intact plants were used which were sprayed with either 2 mM phenidone solution or a control (0.1% Tween 20) solution until run-off one day before the experiment. The plants were allowed to dry and were subsequently introduced into the cages with butterflies.

Surface application of phenidone.

Brussels sprouts plants were sprayed with 2 mM phenidone (1-phenyl-pyrazolidinone, Sigma-Aldrich) in 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma) solution or a control (0.1% Tween 20) solution until run-off. The 4th, 5th and 6th leaves counted from the

[1] The Materials and Methods are mainly based on experiments performed by Bruinsma (unpub. results) and Bruinsma *et al*. (2007), unless indicated otherwise.

base of the plant were infested with either *P. rapae* or *P. brassicae* caterpillars, 5 caterpillars per leaf. After 24 hours, the leaves were cut off the plants and their petioles were placed in a vial with tap water and introduced into the cages with butterflies. For *P. rapae* only, a second experiment was performed where the caterpillars where removed from the leaves just before these were placed inside the cages.

*Systemic uptake of phenidone (*P. rapae *only).*

The 4th, 5th and 6th leaves counted from the base of Brussels sprout plants were cut off the plants and placed directly in a 1 mM phenidone solution or in tap water as a control (Engelberth *et al*. 2001, Koch *et al*. 1999). They were subsequently infested with caterpillars, 24 hours prior to the start of the experiment (Engelberth *et al*. 2001). Thereafter, the leaves were introduced into the cages with butterflies. The amount of phenidone taken up was determined by measuring the amount of phenidone solution for every vessel just before and after the experiment. A control vessel containing only tap water was also placed inside the cages to correct for evaporation.

Butterfly oviposition preference test.

Adult butterflies emerged from pupae in an oviposition cage of 67 x 100 x 75 cm in a greenhouse compartment at 22-24°C and 50-70% RH. Artificial light (sodium vapour lamps, type SON-T, 500W, Philips, The Netherlands) was used in the cage from 8.00 am until 2.00 pm in addition to natural daylight. The butterflies were provided with a 10% sucrose solution to feed on and a Brussels sprouts plant was introduced into the cage to oviposit on.

In each oviposition cage of 67 x 50 x 75 cm, one male and one female butterfly were introduced one day before the experiment. They were provided with 10% sucrose solution to feed on. The next morning, one leaf treated with phenidone solution and one treated with control solution were introduced into the cages. For the experiments to test the different effects of the phenidone solution on the oviposition preference of *P. rapae*, the butterflies were allowed to oviposit from about 8.30 am until 2.00 pm. For the experiments to test the effect of caterpillar infestation and for all experiments using *P. brassicae*, the oviposition behaviour was monitored during 15 minutes (*P. rapae*) or 60 minutes *(P. brassicae*) in the morning. Thereafter, the butterflies were allowed to oviposit without observation of their behaviour for another 4 hours. The leaves were subsequently removed from the cages and the

number of eggs was counted. The experiment was performed using up 5 to 9 cages per day, resulting in a total of 14 to 43 replications. Each day, other pairs of butterflies were used.

Performance of Pieris rapae *caterpillars.*

The performance of *Pieris rapae* caterpillars on phenidone-treated plants and control plants was observed and compared. Brussels sprouts plants were sprayed with a 2 mM phenidone in 0.1% Tween 20 solution or only 0.1% Tween 20 solution as a control. After 24 hours, 30 newly hatched *P. rapae* larvae were evenly distributed over two plants per treatment and placed in double cages of 60x100x75 cm, one cage per treatment, in a greenhouse compartment at 22-24°C and 50-70% RH. The plants were replaced by new plants twice a week, so there was always a maximum of 4 to 5 days of larval feeding on the same plant. The number of days until pupation and the weight of the pupa were measured.

Infested and non-infested plants.

To test the effect of caterpillar infestation, the 4^{th}, 5^{th} and 6^{th} leave of Brussels sprouts plants were infested with five caterpillars per leaf of either *Pieris rapae* or *P. brassicae*. After 24 hours, the leaves were cut off and their petioles were placed in vials with tap water and introduced into the cages with butterflies. For *P. rapae*, this experiment was also performed using 15 caterpillars per leaf, and allowing 5 caterpillars per leaf to feed for 1 week prior to cutting of the leaves and introducing them into the cages with butterflies.

Statistical Analysis

Oviposition preference test without monitoring the behaviour: Each individual butterfly was given a two-choice situation. In case the oviposition data were normally distributed, they were analysed with a paired t-test. In case they are non-normally distributed, they were analysed with a Wilcoxon matched-pair signed-rank test. The data obtained from the caterpillar performance test were analysed with ANOVA in case they were normally distributed. In case of non-normal distribution, they were analyzed with a Mann-Whitney U test for differences between treatments.

Oviposition preference test with monitoring the behaviour: The data consisting of number of eggs, number of batches, number of choices (defined as a landing on a leaf or, in case of *P. rapae*, a series of short landings in a short amount of time during which the butterfly stays

very close to the same leaf) and total time spent on the leaf were analysed with a paired t-test in case of normal distribution or with a Wilcoxon matched-pair signed-rank test in case of non-normal distribution. The data consisting of time until the first choice, time spent on the leaf during the first choice and number of eggs laid during the first choice were analysed with ANOVA in case of normal distribution or with a Mann-Whitney U test for differences between treatments in case of non-normal distribution.

All statistical analyses were done using SPSS 13.0.

Results

Effect of phenidone experiments with *Pieris rapae*

Effect of pure phenidone

Green cardboard paper was sprayed with 5 mM sinigrin solution, followed by either water or 2 mM phenidone solution to test the effect of pure phenidone on the oviposition behaviour of *Pieris rapae* butterflies. The results are shown in Figure 4. No significant difference was found in the number of eggs laid on green cardboard paper sprayed with phenidone solution and the paper sprayed with water (Wilcoxon Signed Ranks test, p = 0.305).

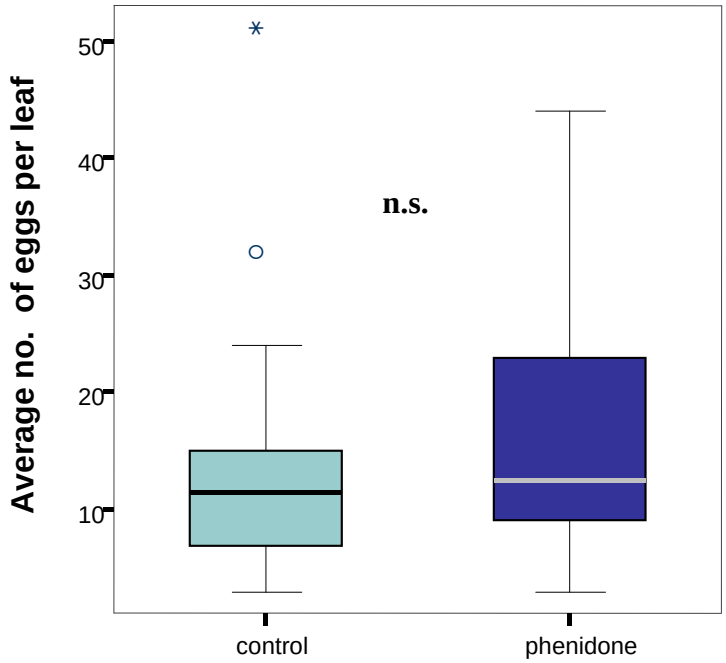

Figure 4: Effect of pure phenidone on the oviposition behaviour of *P. rapae*. Average number of eggs laid on green cardboard paper sprayed with either phenidone or control solution. Wilcoxon Signed Ranks test Z = -1.085; p = 0.305; 14 replications.

Surface application of phenidone

To test the effect of the surface application of phenidone on the oviposition preference of *Pieris rapae* females (Fig. 5), Brussels sprouts plants were sprayed with either phenidone solution or a control solution and subsequently infested with *P. rapae* larvae. The butterflies tended to have a slight preference for the control plants when choosing a suitable host plant.

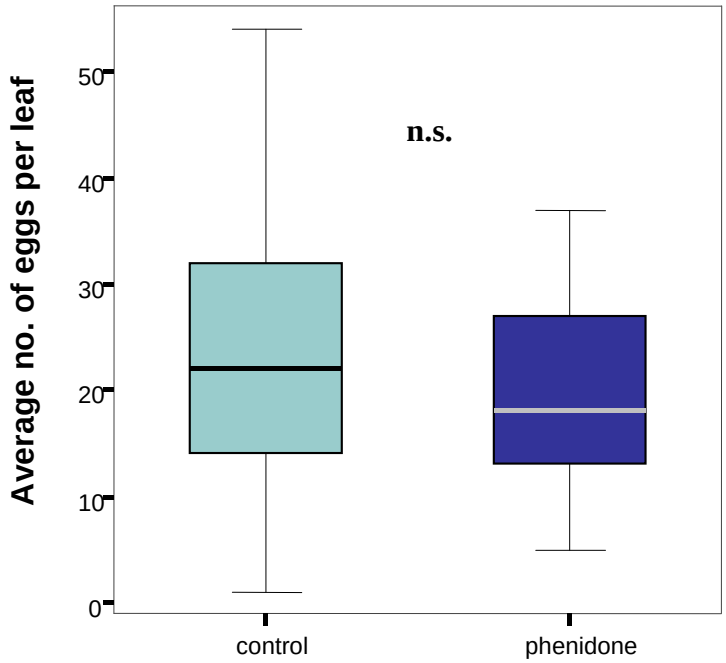

Figure 5: Effect of the surface application of phenidone on the oviposition behaviour of *P. rapae*. Average number of eggs laid on Brussels sprout leaves sprayed with either phenidone or control solution. Wilcoxon Signed Ranks test Z = -1.656; p = 0.099; 33 replications.

However, this difference appeared to be not significant (Wilcoxon Signed Ranks test, p = 0.099).

To determine whether the presence of the larvae on the leaves was the cause of the observed behaviour, the same experiment was repeated, but the larvae where removed from the leaves before these were introduced into the cages. Again, the butterflies appeared to have a slight preference for the control leaves, but the difference was not significant (Wilcoxon Signed Ranks test, p = 0.358) (Appendix 1.1a). When comparing the two different experiments, no significant difference was found in percentage of eggs on the leaves sprayed with control solution as well as the leaves treated with phenidone solution (Mann-Whitney U test, p = 0.856 for both experiments) (Appendices 1.1b and 1.1c). Removal of the larvae was thus considered unnecessary for further experiments.

Systemic uptake of phenidone

Leaves of Brussels sprouts plants were cut off and placed in vials with either 1 mM phenidone solution or water as a control to determine the effect of systemic uptake of phenidone on the oviposition behaviour. No significant difference was observed in number of eggs laid on the

leaves treated with phenidone solution and those treated with tap water (Wilcoxon Signed Ranks test, p = 0.665). The results are shown in Appendix 1.2a.

The phenidone solutions changed colour within 24 hours from transparent orange to dark purple with a black residue on the bottom of the vessels. This residue is possibly not taken up by the leaves. To determine the relation between the amount of phenidone taken up by the leaves and the amount of eggs laid on these leaves, the amount of phenidone solution present in the vessel was measured before and after the experiment and this amount was corrected for evaporation. The correlation between the amount of phenidone taken up and the amount of eggs laid appeared to be weak (Spearman Correlation, p = 0.086) (Appendix 1.2b).

Effect of larval infestation experiments with *Pieris rapae*

Because of the insignificant results of the former experiments, it was hypothesised that *P. rapae* might not differentiate between caterpillar-infested and uninfested plants, contrary to the observations made by Shiojiri *et al.* (2002). The butterflies were given a two-choice situation of clean, uninfested Brussels sprout leaves and leaves from which 5 larvae had been eating for 24 hours. Furthermore, the butterflies were monitored for 15 minutes in order to get more insight in their behaviour. Thereafter, the butterflies were allowed to oviposit for another 4 hours without observation. The difference between the number of eggs laid on the infested and the uninfested leaves turned out to be not significant after the first 15 minutes (Wilcoxon Signed Ranks test, p = 0.390) (Appendix 2.1a). At the end of the experiments, the butterflies seemed to have a preference for uninfested control plants (Fig. 6). However, this effect appeared to be not significant after statistical analysis (Wilcoxon Signed Ranks test, p = 0.093). Furthermore, the butterflies chose to visit both plants just as often within the first 15 minutes (Wilcoxon Signed Ranks test, p = 0.487) (Appendix 2.1b) and no significant difference was found in the amount of time spent before the butterflies made their first choice (Mann Whitney U test, p = 0.391) (Appendix 2.1c). The butterflies seemed to lay more eggs on the infested plants during their first choice (Appendix 2.1d), but these results turned out to be not significant (Mann-Whitney U test, p = 0.572).

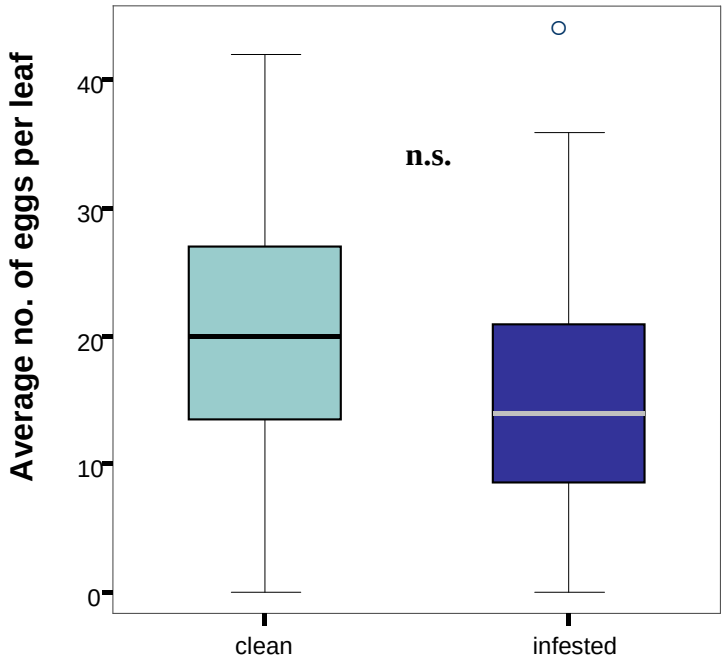

Figure 6: Effect of larval infestation (5 larvae per leaf) on the oviposition preference of *P. rapae*. Average total number of eggs laid on either clean or infested Brussels sprout leaves. Wilcoxon Signed Ranks test Z = -1.679; p = 0.093; 43 replications.

This experiment was repeated using 15 larvae per leaf. Here, the number of eggs on the clean, uninfested leaves was significantly higher than the number of eggs on the leaves infested with caterpillars after the first 15 minutes (Wilcoxon Signed Ranks test, $p = 0.003$) (Appendix 2.2a) as well as at the end of the experiment (Wilcoxon Signed Ranks test, $p < 0.001$) (Fig. 7). Also, significantly more choices were made for the clean leaves than for the infested leaves (Wilcoxon Signed Ranks test, $p = 0.007$) (Appendix 2.2b). However, the amount of time spent until the first choice was made was similar (Mann-Whitney U test, $p = 0.870$) (Appendix 2.2c) and also no significant difference was found in number of eggs laid during the first choice (Mann-Whitney U test, 0.328) (Appendix 2.2d).

This experiment was repeated a second time using 5 larvae per leaf, however this time the larvae were allowed to feed for 1 week before the leaves were cut off and placed inside the cages with butterflies.

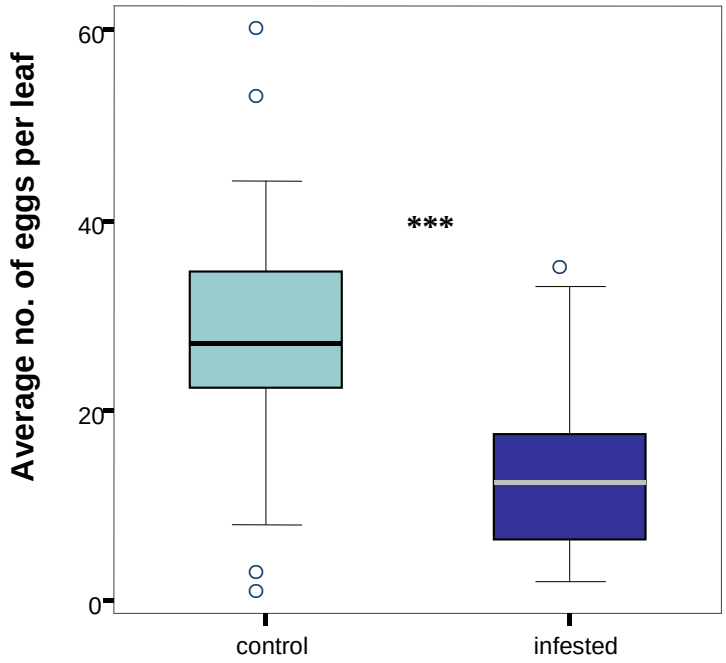

Figure 7: Effect of larval infestation (15 larvae per leaf) on the oviposition behaviour of *P. rapae*. Average total number of eggs laid on either clean or infested Brussels sprout leaves. Wilcoxon Signed Ranks test Z = -3.531; $p < 0.001$; 24 replications.

After 15 minutes, there was no significant difference in number of eggs laid on the infested and the uninfested leaves (Wilcoxon Signed Ranks test, $p = 0.055$) (Appendix 2.3a). However, a significant difference was found in number of eggs at the end of the experiment (Wilcoxon Signed Ranks test, $p = 0.004$) (Fig. 8). Furthermore, significantly more choices were made for the clean leaves than for the infested leaves (Wilcoxon Signed Ranks test, $p = 0.006$) (Appendix 2.3b). No significant difference was observed in the amount of time spent before the first choice was made (Wilcoxon Signed Ranks test, $p = 0.682$) (Appendix 2.3c) or the number of eggs laid within the first choice (Wilcoxon Signed Ranks test, $p = 0.422$) (Appendix 2.3d).

After one week of feeding, the *P. rapae* larvae had greatly increased in size and were dispersed to other parts of the plant. This means that, at the moment the leaves were introduced into the cages with butterflies, often less than 5 larvae were present on the leaf. In 16 out of 24 cases, no larvae were present on the leaf during the experiment.

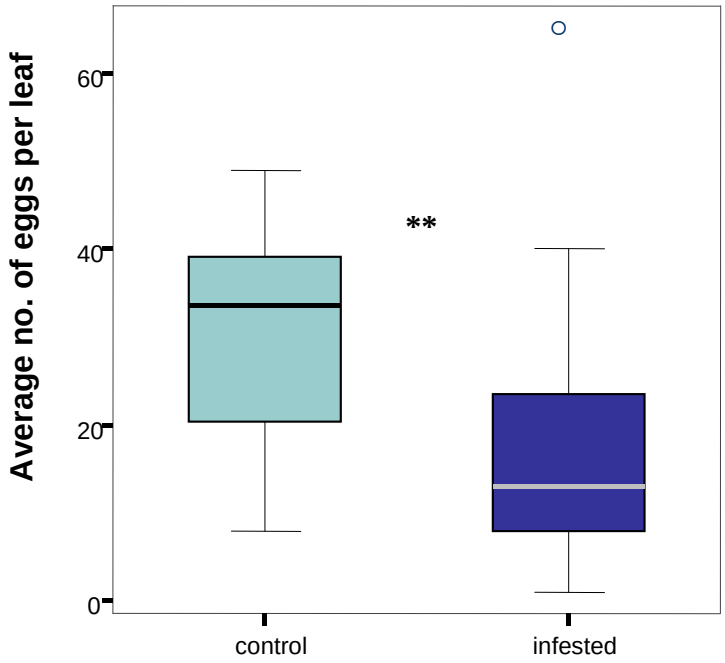

Figure 8: Effect of larval infestation (1 week of damage) on the oviposition behaviour of *P. rapae*. Average total number of eggs laid on either clean or infested Brussels sprouts leaves. Wilcoxon Signed Ranks test Z = -2.799; p = 0.004; 24 replications.

Experiments with phenidone were not performed using 15 larvae per leaf or 5 larvae per leaf with one week of feeding damage, as a density of 15 larvae per leaf is not a good representation of the natural situation and because of the problems with larval dispersion when allowing larvae to feed for 1 week. Also, it is not known whether the phenidone solution might lose its function after several days.

Effect of phenidone on the performance of *Pieris rapae* caterpillars

To determine the effect of phenidone on the performance of *Pieris rapae* caterpillars, two Brussels sprouts plants were sprayed with either water of 2 mM phenidone solution and caterpillars were allowed to feed from these plants until pupation. No significant difference was found between the number of days until pupation for larvae that had been feeding on plants sprayed with phenidone solution and larvae that had been feeding on control plants (Mann-Whitney U test, p = 0.798) (Fig. 9). Also, no significant difference was found in pupa weight (Mann-Whitney U test, p = 0.990) (Fig. 10).

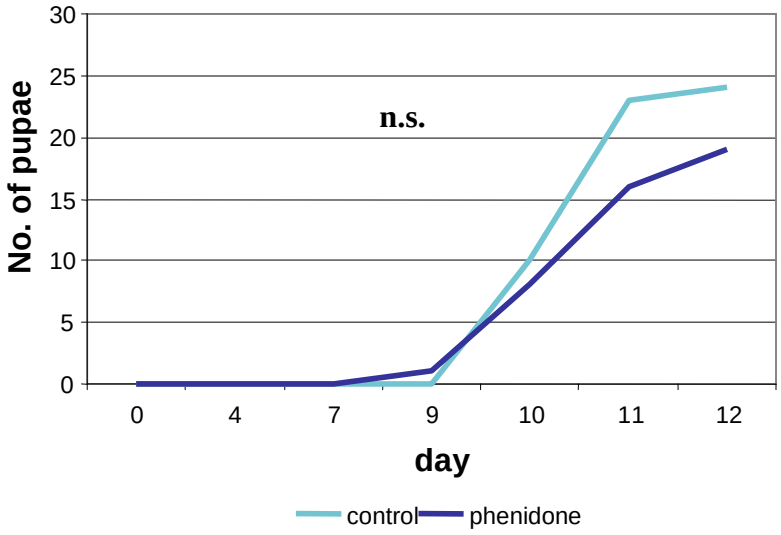

Figure 9: Effect of phenidone on the performance of *P. rapae* larvae. Number of days until pupation. Cumulative number of pupae on each day after the newly hatched larvae were placed on plants sprayed with either phenidone or control solution. Mann-Whitney U test Z = -0.256; p = 0.798; 24 and 19 replications respectively.

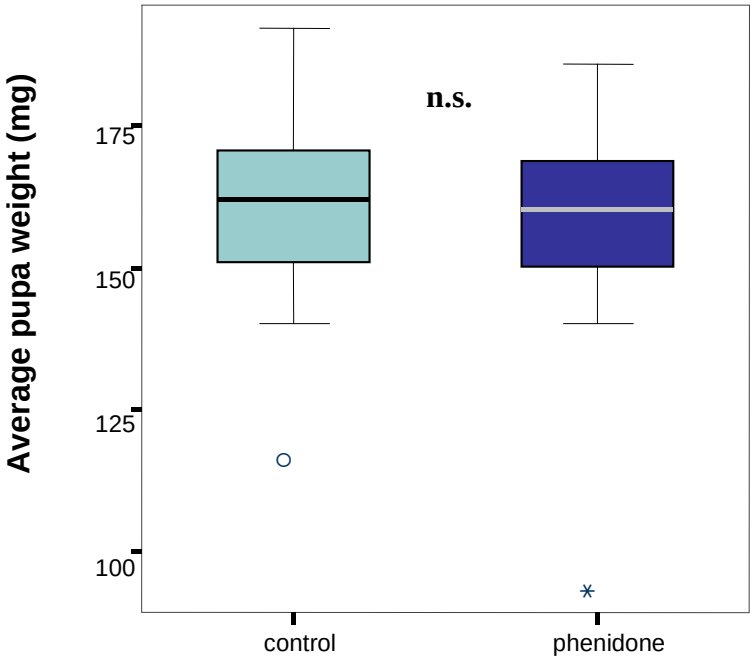

Figure 10: Effect of phenidone on the performance of *P. rapae* caterpillars. Average pupae weight after larvae had been feeding on plants sprayed with either phenidone or control solution. Mann-Whitney U test Z = -0.024; p = 0.990; 24 and 19 replications respectively.

Six larvae for control and ten larvae for phenidone have died or are escaped. One larvae that had been feeding on phenidone-sprayed plants still had not pupated at day 24.

Effect of larval infestation experiments with *Pieris brassicae*

Because of the results obtained from the experiments with *Pieris rapae*, it was decided to start with experiments to determine the effects of larval infestation for *Pieris brassicae*. The experimental design was similar as for the effect of larval infestation experiments with *Pieris rapae*, using 5 *P. brassicae* larvae per leaf. However, the butterflies were monitored for 60 minutes instead of 15, as *P. brassicae* spends more time ovipositing on the leaves than *P. rapae*. The amount of eggs after the first 60 minutes had to be estimated, as counting the exact number of eggs could cause the eggs to break or fall off the leaf.

After the first 60 minutes, the butterflies had shown a significant preference for the clean leaves over the infested leaves. More eggs had been laid on the clean leaves than on the infested leaves (Wilcoxon Signed Ranks test, p = 0.007) (Appendix 3a) and the butterflies chose to visit the clean leaves more often than the infested leaves (Wilcoxon Signed Ranks test, p = 0.004) (Appendix 3b). Furthermore, significantly more time had been spent on the clean leaves than on the infested leaves (Appendix 3c). There was still a significant difference in number of eggs laid on the clean leaves and the infested leaves at the end of the experiment (Wilcoxon Signed Ranks test, p = 0,001) (Fig. 11).

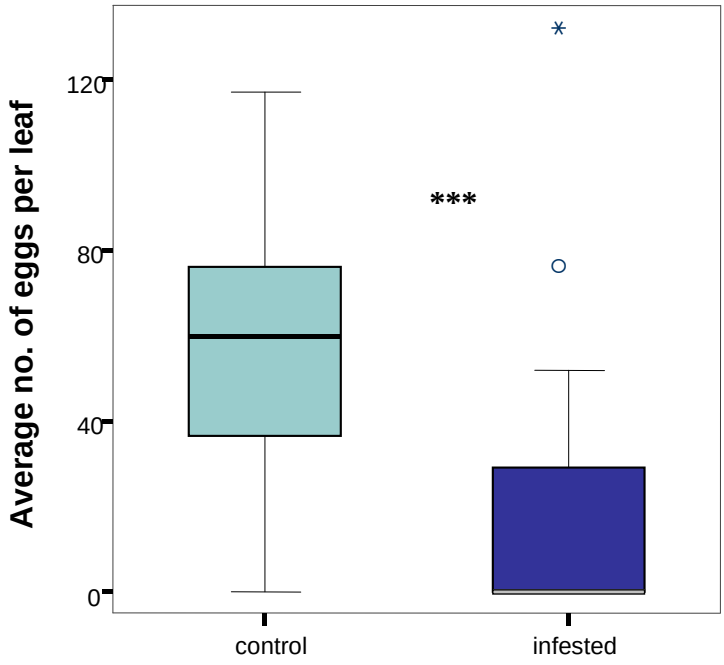

Figure 11: Effect of larval infestation (5 larvae per leaf) on the oviposition preference of *P. brassicae*. Average total number of eggs laid on either clean or infested Brussels sprouts leaves. Wilcoxon Signed Ranks test Z = -3.244; p = 0.001; 31 replications.

No significant difference was found in the amount of time spent until the first choice was made (Mann-Whitney U test, p = 0.421) (Appendix 3d), however, the butterflies spent more time on the clean leaves during their first choice than on the infested leaves (Mann-Whitney U test, p = 0.014) (Appendix 3e).

Effect of phenidone experiments with *Pieris brassicae*

Effect of pure phenidone

To investigate whether pure phenidone influences the oviposition behaviour of *P. brassicae* butterflies, intact Brussels sprouts plants were sprayed with either 2 mM phenidone solution or a control solution. Intact plants were used instead of cardboard paper in order to get a better representation of *P. brassicae*'s natural oviposition habitat.

No significant difference in number of eggs (Wilcoxon Signed Ranks test, p = 0.992) (Appendix 4.1a) or number of batches (Wilcoxon Signed Ranks test, p = 0.563) (Appendix 4.1b) was found after the first 60 minutes. Furthermore, the butterflies did not show a preference for either the plants sprayed with phenidone solution or the control plants in terms of choices (Wilcoxon Signed Ranks test, p = 0.798) (Appendix 4.1c) and they spent an equal amount of time on both plants within the first 60 minutes (Wilcoxon Signed Ranks test, p = 0.974) (Appendix 4.1d). At the end of the experiments, there was still no significant difference in number of eggs (Wilcoxon Signed Ranks test, p = 0.842) (Fig. 12) or number of batches (Wilcoxon Signed Ranks test, p = 0.399) (Fig. 13). Also, no significant difference was found in the amount of time spent before the first choice was made (Mann-Whitney U test, p = 0.395) (Appendix 4.1e) and the butterflies spent an equal amount of time on the plant during their first choice (Mann-Whitney U test, p = 0.698) (Appendix 4.1f).

Effect of larval infestation with phenidone-sprayed plants

This experiment was similar as the effect of larval infestation experiment, however, plants sprayed with 2 mM phenidone solution were used instead of unsprayed plants, to determine whether treatment with phenidone would influence the butterflies' preference for uninfested plants negatively.

After the first 60 minutes, there seemed to be a higher number of eggs laid on the clean leaves than on the leaves infested with larvae, but this effect was not significant (Wilcoxon Signed Ranks test, p = 0.060) (Appendix 4.2a).

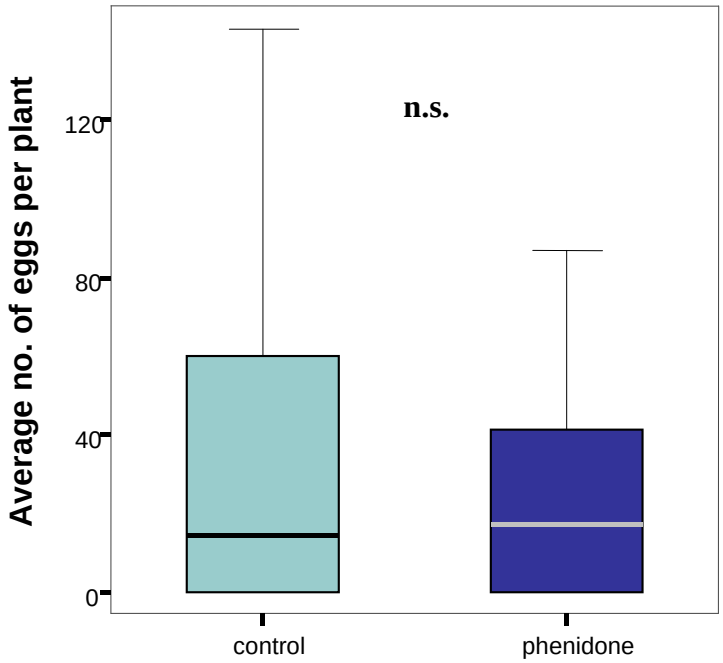

Figure 12: Effect of pure phenidone on the oviposition behaviour of *P. brassicae*. Average total number of eggs laid on Brussels sprouts plants sprayed with either phenidone or control solution. Wilcoxon Signed Ranks test Z = -0.211; p = 0.842; 22 replications.

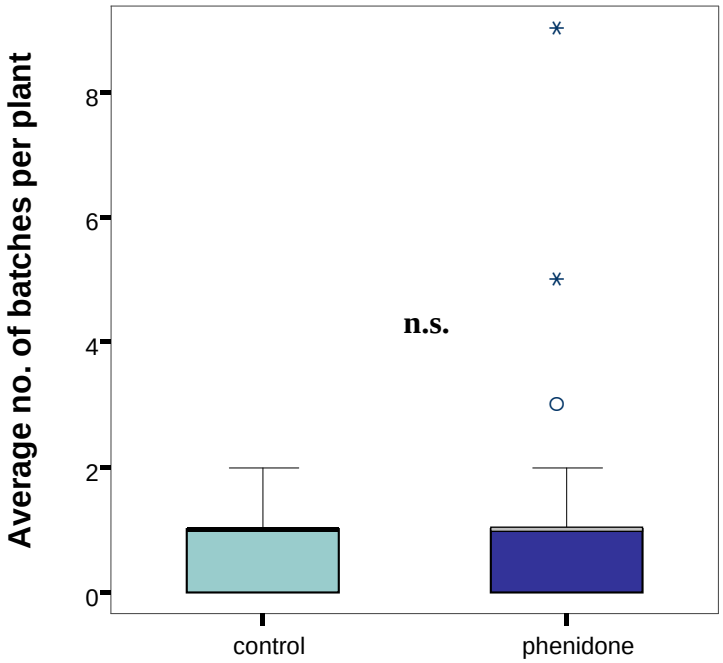

Figure 13: Effect of pure phenidone on the oviposition behaviour of *P. brassicae*. Average total number of batches found on Brussels sprouts plants sprayed with either phenidone or control solution. Wilcoxon Signed Ranks test Z = -0.241; p = 0.399; 22 replications.

However, significantly more batches were found on the clean leaves than on the leaves infested with larvae (Wilcoxon Signed Ranks test, p = 0.015) (Appendix 4.2b).

The butterflies did not discriminate between infested and uninfested plants when making a choice for a certain leaf (Wilcoxon Signed Ranks, p = 0.131) (Appendix 4.2c) but they did spend significantly more time on the clean leaves than on the leaves infested with larvae during the first 60 minutes (Wilcoxon Signed Ranks test, p = 0.034) (Appendix 4.2d).

At the end of the experiment, the difference in number of eggs was still not significant (Wilcoxon Signed Ranks test, p = 0.058) (Fig. 14), while there was still a significant difference in number of batches (Wilcoxon Signed Ranks test, p = 0.005) (Fig. 15).

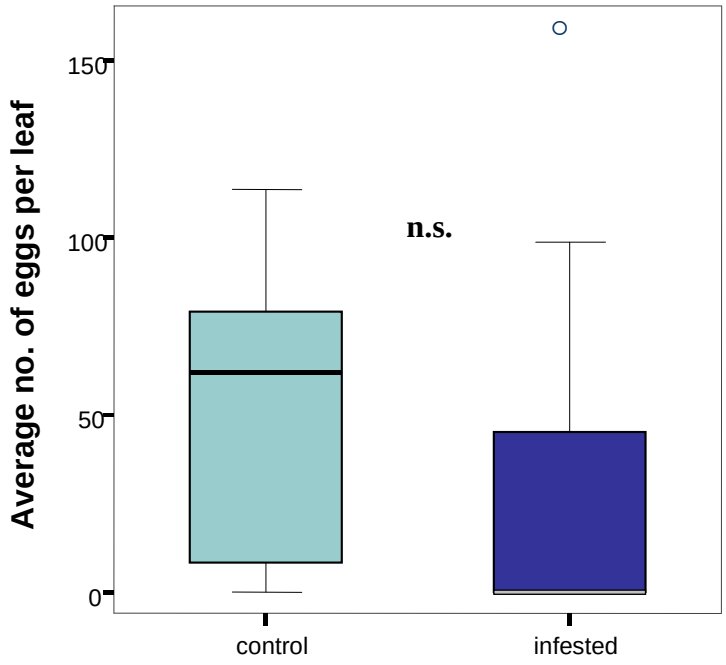

Figure 14: Effect of larval infestation (5 larvae per leaf) with phenidone-sprayed Brussels sprouts plants on the oviposition behaviour of *P. brassicae*. Average total number of eggs laid on clean or infested leaves. Wilcoxon Signed Ranks test Z = -1.894; p = 0.058; 33 replications.

No significant difference was found in the amount of time the butterflies spent until making their first choice (Mann-Whitney U test, p = 0.673) (Appendix 4.2e). They seemed to spend more time on the clean leaf than on the infested leaf during their first choice, but this difference turned out to be insignificant (Mann-Whitney U test, p = 0.070) (Appendix 4.2f).

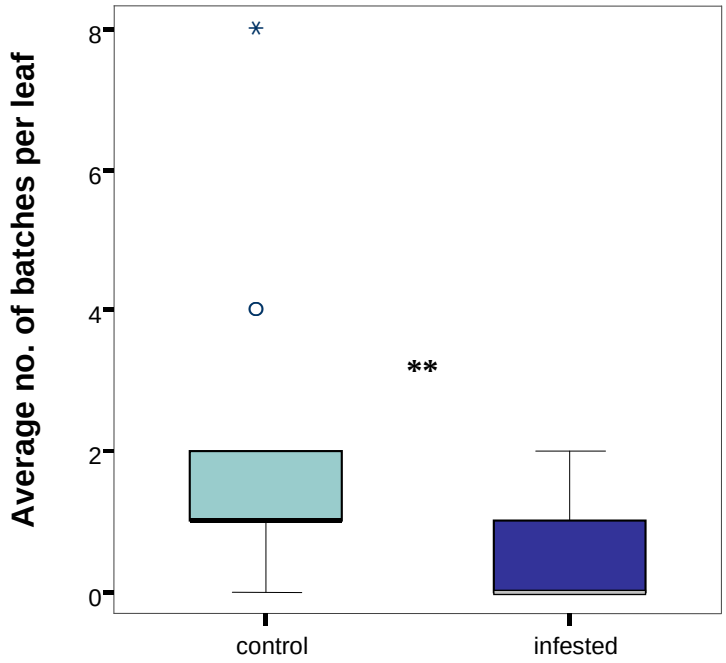

Figure 15: Effect of larval infestation (5 larvae per leaf) with phenidone-sprayed Brussels sprouts plants on the oviposition behaviour of *P. brassicae*. Average total number of batches found on either clean or infested leaves. Wilcoxon Signed Ranks test Z = -2.784; p = 0.005; 33 replications.

When comparing the results of this experiment with the effect of larval infestation experiment with unsprayed plants (Appendices 4.2g and 4.2h), no significant difference was found in percentage of eggs for both clean and infested leaves (Mann-Whitney U test, p = 0.952 for both experiments).

Effect of the surface application of phenidone with infested plants.

Brussels sprouts plants were sprayed with either 2mM phenidone solution or a control solution and subsequently, both were infested with larvae to test the effect of the surface application of phenidone on the oviposition behaviour of *P. brassicae*. After the first 60 minutes, the butterflies seemed to have laid more eggs on the control leaves. However, these results turned out to be not significant (Wilcoxon Signed Ranks test, p = 0.209) (Appendix 4.3a). Also, no significant difference was found in number of batches after 60 minutes (Wilcoxon Signed Ranks test, p = 0.370) (Appendix 4.3b) and the butterflies did not show a preference for control leaves or phenidone-sprayed leaves in terms of choices (Wilcoxon Signed Ranks test, p = 0.770 (Appendix 4.3c). Furthermore, the butterflies spent an equal amount of time on both leaves during the first 60 minutes (Wilcoxon Signed Ranks test, p = 0.370) (Appendix 4.3d).

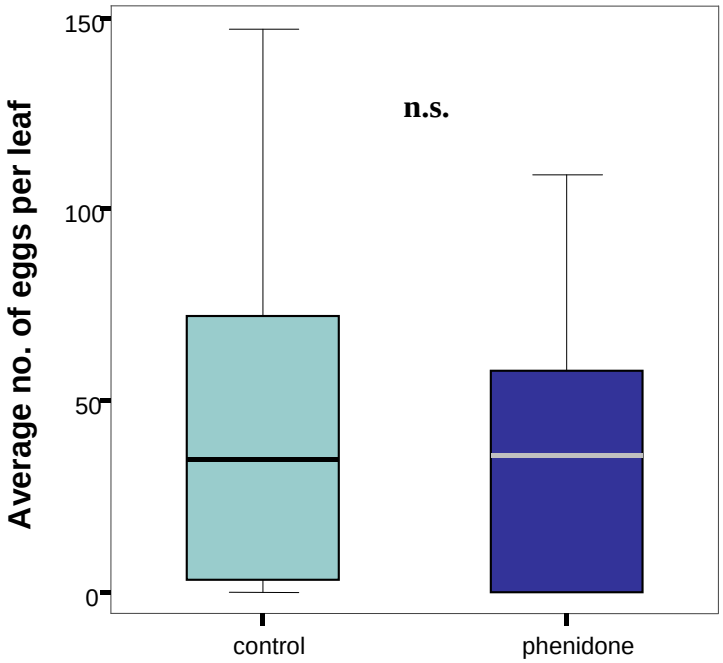

Figure 16: Effect of the surface application of phenidone on the oviposition behaviour of *P. brassicae*. Average total number of eggs laid on Brussels sprout leaves sprayed with either phenidone or control solution. Wilcoxon Signed Ranks test Z = -0.573; p = 0.573; 36 replications.

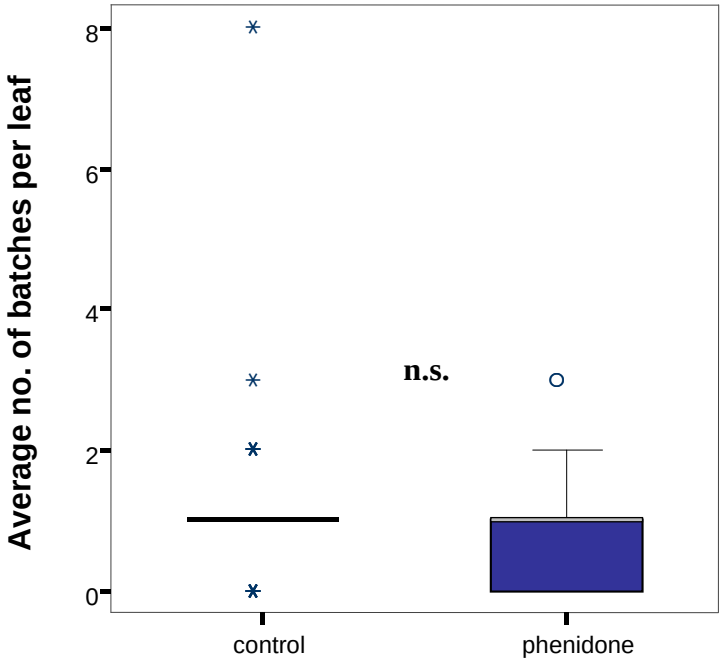

Figure 17: Effect of the surface application of phenidone on the oviposition behaviour of *P. brassicae*. Average total number of batches found on Brussels sprout leaves sprayed with either phenidone or control solution. Wilcoxon Signed Ranks test Z = -1.108; p = 0.282; 36 replications.

At the end of the experiment, there was even less difference in number of eggs laid (Wilcoxon Signed Ranks test, $p = 0.573$) (Fig. 16).

No significant difference in number of batches was found at the end of the experiment (Wilcoxon Signed Ranks test, $p = 0.282$). An equal amount of time was spent until the butterflies made their first choice (Mann-Whitney U test, $p = 0.440$) (Appendix 4.3e). They seemed to spend more time on the control leaves during the first choice, but this difference was not significant (Mann-Whitney U test, $p = 0.271$) (Appendix 4.3f).

Discussion

Effect of phenidone treatment of Brussels sprouts plants on *Pieris rapae* oviposition

Pieris rapae butterflies did not discriminate between green cardboard sprayed with either phenidone or a control solution. They did thus not react to phenidone solution itself (Fig. 4) which means it can be assumed that the observed effect of the other experiments with phenidone are caused by the effect of phenidone on processes in the plant, and not by the compound itself.

Phenidone did not seem to have any effect on the oviposition behaviour of *Pieris rapae* butterflies. Rotschild *et al*. (1977) discovered that *P. brassicae* butterflies discriminate between clean host plants and plants on which larvae have been feeding. Sato *et al*. (1999) found a similar response for *P. rapae* and Bruinsma *et al*. found that both *Pieris* species discriminate between leaves sprayed with either jasmonic acid or a control solution. It was therefore hypothesised that *P. rapae* would have a preference for leaves treated with phenidone. However, the butterflies did not show a preference for either treatment when choosing an oviposition site (Fig. 5, Appendix 1.2a). When having a choice between control leaves and leaves sprayed with phenidone solution, it was investigated whether larval presence could be an explanation for the observed effect. There could be a possibility that the butterflies have a preference for leaves treated with phenidone, as this compound is known to interfere with the octadecanoid pathway, but change their decision when they detect feeding larvae on the leaf. However, the results showed no difference with the original experimental design (Appendices 1.1a, 1.1b and 1.1c). It was preferred to continue with the original experimental design, as it allowed larvae to continue feeding for another few hours and thus continue inducing the host plant's defence system.

Apart from the amount of eggs per leaf, the relation between the amount of eggs laid and the amount of phenidone taken up by the leaf was measured when the butterflies had a choice between leaves standing in vessels with either tap water or phenidone solution. The results showed a weak relation (Appendix 1.2b). There could be several four to explain this observation. First, it could be possible that phenidone does not inhibit LOX activity and thus does not interfere with the octadecanoid pathway. This is however unlikely, as other studies have proven phenidone to be an efficient inhibitor of LOX (Engelberth *et al*. 2001, Koch *et al*. 1999, Paré et al. 1996). Second, phenidone might not be taken up by the cabbage leaf in amounts high enough to interfere with LOX activity. This could be a more likely explanation, as the colour of the phenidone solution changed from orange to dark purple with a black

residu on the bottom of the vessel within 24 hours. It is expected that phenidone is no longer taken up by the leaves when this black residu was present. Third, the changes in leaf chemistry caused by phenidone could be too small to be detected by and induce a response in ovipositing *Pieris rapae* females. This could also be an explanation for the effect observed when the butterflies had to choose between leaves sprayed with either phenidone solution or control solution. And fourthly, most likely, the butterflies could not be making a difference between infested and uninfested plants and thus any changes caused by phenidone will not make any difference in the behaviour of the butterflies.

Effect of larval infestation on *Pieris rapae* oviposition

Poelman (unpub. data) discovered that *P. rapae* butterflies did not discriminate between undamaged leaves or leaves of which *P. rapae* larvae had been feeding when choosing an oviposition site for two white cabbage cultivars (Badger-Shipper and Rivera). It was therefore hypothesised *P. rapae* butterflies might also not discriminate between undamaged leaves and leaves infested with caterpillars for the Brussels sprouts cultivar used in these experiments: Cyrus.

When applying five larvae per leaf, on three leaves per plant for 24 hours, the results showed that *P. rapae* butterflies did indeed not discriminate between clean, undamaged leaves and leaves of which caterpillars had been feeding (Fig. 6). These results provide a plausible explanation for the results obtained from the experiments with phenidone. Chemical changes in the leaf caused by phenidone will have no effect on the oviposition behaviour of the butterflies if they do not show a preference for undamaged leaves.

However, when either applying 15 larvae per leaf or allow 5 larvae per leaf to feed for a week, the butterflies showed a preference for the uninfested leaves (Fig. 7, 8). This effect was stronger after the butterflies had had more time to oviposit (Appendix 2.2a, Fig. 7, Appendix 2.3a and Fig. 8 respectively).

The butterflies' behaviour was monitored for 15 minutes for all larval infestation experiments. The number of choices (defined as a series of short landings in a short amount of time during which the butterfly stays very close to the same leaf) the butterflies made were positively related to the number of eggs laid, as expected. In none of the cases did the butterflies show a preference for one of the two treatments when looking at the amount of time spent before they made their first choice and the number of eggs they laid during their first choice.

This means the butterflies explore both plants equally during the first minutes. Their preference shows in the amount of times they visit a certain leaf and probably not by laying more eggs on the preferred leaf during one choice. During the first 15 minutes, the butterflies often showed a flying pattern in which they alternated fast between the two different leaves (results not shown).

These results suggest *P. rapae* butterflies rather spread their eggs over different plants than choose a single, best suitable host plant, unless a very large number of larvae was present on the leaf or the larvae had been feeding on the leaf for a week, which both resulted in a lot of leaf damage. However, such a large amount as 15 larvae per leaf is very unlikely to be found in the natural situation, as *P. rapae* butterflies lays single eggs. The experiment where larvae were allowed to feed for a week is a better representation of the natural situation, however, the data obtained from this experiment might not be a hundred percent accurate, as in most cases, the larvae had dispersed to different parts of the plant after one week and no larvae were present on the leaf during the experiment. Furthermore, the butterflies showed no preference for one of the two leaves during the first 15 minutes. As *P. rapae* lays its eggs singly and therefore exploits more than one host plant, they will usually not spend more than 15 minutes near the same host plant in natural situations. For these reasons, the experiments to test the effect of phenidone on the oviposition behaviour of *P. rapae* were not repeated using either 15 larvae per leaf or allowing 5 larvae per leaf to forage for a week.

According to Davies and Gilbert (1985), *P. rapae* butterflies can be less discriminatory than gregarious species when choosing a suitable host plant, as they lay single eggs on different, often isolated host plants. Root and Kareiva (1984) observed flight patterns of *P. rapae* that causes an even spread of eggs. They called this the “egg-spreading syndrome”. This explains the behaviour observed when the butterflies had a choice between clean leaves and leaves on which 5 larvae had been feeding for 24 hours, where the butterflies preferred to lay their eggs on both leaves and alternated fast between the two leaves, instead of choosing one, most suitable host plant. The results show that the butterflies will only prefer undamaged host plants when the alternative leaves are highly damaged or contain an unrealistically high number of herbivores.

Effect of phenidone on the performance of *Pieris rapae* caterpillars.

If the octadecanoid pathway would be successfully blocked by phenidone, the larvae feeding

from phenidone-sprayed plants would not be exposed or be exposed to a lesser extent to plant defence compounds that would have a negative influence on their growth. It was therefore expected that *Pieris rapae* caterpillars that had been feeding on leaves sprayed with phenidone solution would grow faster and obtain a higher pupa weight than caterpillars that had been feeding from plants sprayed with control solution. However, the results show no significant difference in number of days until pupation (Fig. 9) or average pupal weight (Fig. 10) between the two treatments. The results might not be accurate as one third of the larvae that had been feeding on phenidone-sprayed plants could not be measured due to death, disappearance and a larva that did not pupate. Another explanation for the observed effect could be that new plants were provided every 4 to 5 days, while the effect of phenidone has not been tested after longer than 24 hours. If phenidone does not lose its function and the changes in leaf chemistry are strong enough to affect the larvae, there is a possibility that the larvae do benefit from phenidone activity, but might experience a negative effect because of the compound itself. It was not investigated whether phenidone is toxic for *P. rapae* caterpillars.

Effect of larval infestation on *Pieris brassicae* oviposition

Contrary to the results obtained when *P. rapae* had a choice between clean leaves and leaves infested with caterpillars, *P. brassicae* did discriminate between clean and infested leaves. Figure 11 shows that the butterflies have a strong preference for ovipositing on clean leaves over leaves infested with five larvae. This was as expected, as *P. brassicae* lays eggs in clusters of 10 to 150 eggs at a time. Spending so much time as well as leaving so much offspring on one single plant makes proper host selection a lot more important for *P. brassicae* than for *P. rapae*, who prefers to spread its eggs. *P. brassicae* often visited both leaves at least once but returned more often to the preferred leaf. No significant difference was observed in the amount of time that was spent before the butterflies chose to visit either of the two leaves (Appendix 3d), however, they did spend significantly more time on the clean leaves during the first choice (appendix 3e) including oviposition. This suggests the butterflies either accept or reject a potential host plant upon contact, as was found by Renwick and Radke (1988), Tabashnik (1987), Huang and Renwick (1993) and Renwick and Chew (1994) for *Pieris rapae*.

Although an amount of five larvae per leaf might be lower than in the natural situation, it was considered unnecessary to repeat the experiment with a higher number of larvae as *P. brassicae* already detects a difference at lower densities.

Effect of phenidone treatment of Brussels sprouts plants on *Pieris brassicae* oviposition

The results of the *Effect of pure phenidone*-experiment showed that *P. brassicae* does not discriminate between plants sprayed with phenidone and plants prayed with control solution (Fig. 12, Appendix 4.1a). It can thus be assumed the observed effect of the other experiments with phenidone are not caused by phenidone itself, but by the effect on the plant caused by treatment with this compound.

As *P. brassicae* reacts stronger to the chemical changes in the leaf induced by herbivores than *P. rapae*, there was a possibility that the butterflies would also be more sensitive to the chemical changes in the leaf induced by the application of phenidone. First, the experiment in which the butterflies had to choose between clean leaves and leaves infested with larvae was repeated, but this time using plants sprayed with phenidone instead of unsprayed plants. To the contrary of the observed effect when using unsprayed plants, there was no significant difference in number of eggs between the infested and the clean leaves (Fig. 14, appendix 4.2a), although more egg batches were found on the clean leaves (Fig. 15, appendix 4.2b). This suggest there must be some effect of phenidone on the oviposition behaviour of *P. brassicae*. It is also interesting to see that the butterflies chose to visit both plants an equal amount of times as can be found in appendix 4.2c, but spent more time on the clean leaves (Appendix 4.2d). Still, no significant difference in number of eggs between both treatments was founds. This means the butterflies laid more eggs per batch and within a shorter time on the infested leaves than on the uninfested leaves.

However, when comparing the number of eggs found at the end of the experiment where butterflies had to choose between infested and uninfested leaves using unsprayed plants, and the similar experiment using phenidone-sprayed plants for both treatments, no significant differences were found (appendices 4.2g and 4.2h). The experiments could be compared as the number of replications was almost equal (31 for when using unsprayed plants versus 33 for when using phenidone-sprayed plants) so it is unlikely that this result was caused by chance. Although the difference in number of eggs was not significant when using phenidone-sprayed plants, it showed a quite strong tendency towards clean plants ($p = 0.058$) at the end of the experiment. Phenidone treatment thus has some effect on the oviposition preference of *P. brassicae*, but it does not seem to be very strong.

The results obtained when the butterflies had to choose between infested plants sprayed with control solution and infested plants sprayed with phenidone solution did not show any significant differences for any of the observed aspects between the two different treatments.

This suggest that the inhibition of chemical changes in the leaf surface induced by phenidone are not strong enough for *P. brassicae* to be detected when the potential host plants both have feeding damage.

Conclusion

According to the obtained results, *P. rapae* butterflies do not discriminate between clean plants and herbivore-infested plants when choosing an oviposition site. According to Sato (1999), *P. rapae* does prefer clean *Rorippa indica* plants over infested plants, but this research used very few replications and the results may thus not be accurate. Poelman (unpub. data) used more than 20 replications, as in this research, and found no difference in number of eggs between clean and infested plants cabbage plants. It can be assumed *P. rapae* butterflies consider an even spread of eggs to be more important than finding a single, most suitable host plant. Shiojiri *et al*. (2002) found that *P. rapae* did also not discriminate between clean plants and plants infested with *Plutella xylostella* larvae, although the presence of these larvae increase the risk of just hatched *P. rapae* larvae to be parasitized by the parasitoid wasp *Cotesia glomerata*. According to Shiojiri *et al*. (2002), *P. rapae* avoids parasitism by moving to a habitat where the parasitoid is absent. It could be that *P. rapae* uses the same strategy when coming across plants infested with larvae of its own species. *P. brassicae*, however, lays large amounts of eggs on the same host plant in clumped vegetation and thus will face more risk of competition and parasitation for their larvae if they do not select a suitable host plant.

The egg-spreading strategy of *P. rapae* is likely to be one of the explanations as for why this species does not discriminate between infested control plants and infested plants treated with phenidone. This explanation does not go for *P. brassicae* however. Although a small effect was observed of the compound when the butterflies were given a choice between infested and uninfested leaves using phenidone-sprayed plants, there was no significant difference with the experiment in which the butterflies had to choose between infested and unifested plants using unsprayed plants. Furthermore, no difference was observed when *P. brassicae* was given a choice between infested control leaves and infested leaves sprayed with phenidone solution. The observed change in colour of the phenidone solution after several hours could mean the compound loses its function after several hours. This is however unlikely, as similar concentration and way of phenidone application were used in two other studies on Brussels sprouts plants by Bruinsma (thesis 2008) who investigated the preference

of different parasitoid wasp species for *P. rapae*-infested plants sprayed with either a control solution or phenidone solution, and Bustos Salvador and Van den Biggelaar (student report 2007) who investigated the preference of *Plutella xylostella* on *P. rapae*-infested plants sprayed with either control solution or phenidone solution. Bruinsma found that *Cotesia glomerata* landed significantly more often on herbivore-infested plants treated with control solution than on herbivore-infested plants treated with phenidone solution. The results of Bustos Salvador and Van den Biggelaar showed that *Plutella xylostella* moths preferred to oviposit on leaves damaged by *P. rapae* larvae when using unsprayed leaves, but had no preference when the leaves were treated with phenidone solution. This proves phenidone does not completely lose its function after 24 hours. There must be another reason as to why phenidone has little to no effect on the oviposition behaviour of *Pieris* butterflies.

Kessler and Baldwin (2002) stated that not all LOX isoforms are involved in JA-synthesis. Indeed, Hlasta *et al*. (1991) and Cucurou *et al*. (1991) found that phenidone did also interfere with LOX of animal origin. The possibility that phenidone did in this case interfere with another LOX and not LOX involved in JA-sysnthesis is however unlikely, as phenidone did interfere with JA-synthesis in the experiments of Bruinsma (thesis 2008) and Bustos Salvador and Van den Biggelaar (student report 2007).

Another possible explanation could be that *Pieris* butterflies are less sensitive to the amount of change in leaf chemistry caused by phenidone than *C. glomerata* and *Pl. xylostella*, as Poelman (unpub. data) also observed *Pl. xylostella* significantly prefers leaves damaged by *P. rapae* caterpillars over undamaged leaves. According to Kim *et al*. (2003), wound-induced expression of LOX was not completely inhibited by phenidone, but only delayed for several hours. They did however use a lower concentration of phenidone. If LOX expression was also delayed in this research, which would lead to delayed JA production, this could provide another explanation, apart from phenidone being inactive or being toxic, as to why *P. rapae* caterpillars did not have any benefit when feeding on phenidone-sprayed plants opposed to feeding on plants sprayed with control solution. The larvae would still be affected by the plant defence compounds, only several hours later. Furthermore, if LOX expression is only delayed and not completely blocked, there could be still enough production of plant defence compounds to be detected by *P. brassicae* butterflies. However, as *C. glomerata* heavily relies on the perception of plant volatiles in order to find it's host, it is more likely that LOX expression is indeed delayed and the resulting difference in amount of plant defence compounds produced is strong enough to be detected by *C. glomerata* and *Pl. xylostella*, but not strong enough to be detected by *Pieris* butterflies.

Future research

A lot of questions still remain after this research. It would therefore be interesting to continue investigating this topic. Phenidone, in this concentration, has proven to be an efficient blocker, or at least delayer, of LOX activity which is crucial in the production of plant defence compounds mediated by the octadecanoid pathway in several studies. Engelberth *et al*. (2001) observed that Lima bean plants did not emit any volatiles induced by the octadecanoid pathway when treated with phenidone. Kim *et al*. (2003) found that wound-induced expression of LOX was delayed in plants upon phenidone treatment and phenidone caused the inhibition of extrafloral nectar production induced by the octadecanoid pathway in *Macaranga tanarius* (Heil *et al*. 2001) However, no clear effect was detected by *P. rapae* and *P. brassicae*. As the egg spreading syndrome was the most plausible explanation for the lack of effect in case of *P. rapae*, it would be interesting to try a similar experiment with phenidone allowing a few larvae per leaf to feed for a longer period than a day. Furthermore, higher concentrations of phenidone could be used for the experiments with both butterflies to see if a more efficient blocking of LOX activity can be achieved. Moreover, the effect of phenidone on the performance of caterpillars could also be tested for *P. brassicae*. More plants will be needed for this experiments, as *P. brassicae* larvae consume almost three times the amount of leaf area *P. rapae* consumes. An experiment to test whether phenidone itself is toxic for *Pieris* caterpillars will be useful to rule out this possibility.

If phenidone fails to cause any effect on the oviposition preference of *Pieris* butterflies, different compounds could be tested which also interfere with the octadecanoid pathway. For example, DIECA inhibits allene oxide synthase activity, which is the step after LOX activity in the octadecanoid pathway and transforms 13S-hydroperoxy linolenic acid into 12,13S-epoxyoctadeca-9,11,15-trienoic acid, while *n*-propyl gallate interferes with the next step: allene oxide cyclase, which transforms 12,13S-epoxyoctadeca-9,11,15-trienoic acid into 12-oxo-phytodienoic acid (PDA) (Fig. 3, Koch *et al*. 1999). Measuring of JA levels can be used to test whether these inhibitors, including phenidone, really inhibit JA-synthesis or delay it and to what extent.

These experiments will help to gain more understanding of the effect inhibiting the plant defence system can have on the oviposition behaviour of *Pieris* butterflies, and will thus provide more insight about the complex interactions between plants and herbivorous insects.

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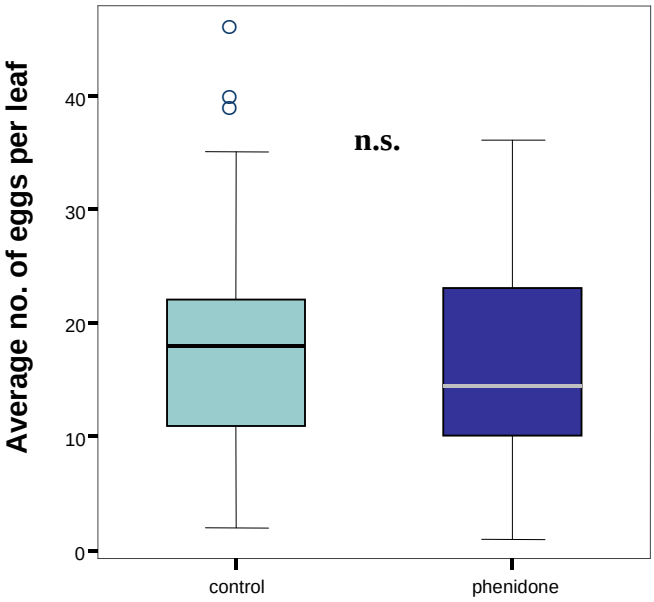
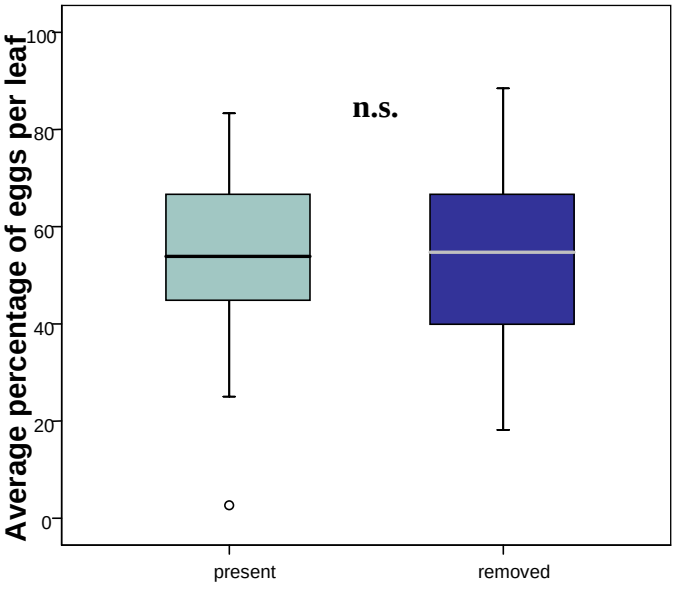
Appendix 1 Effect of phenidone experiments with *Pieris rapae*.

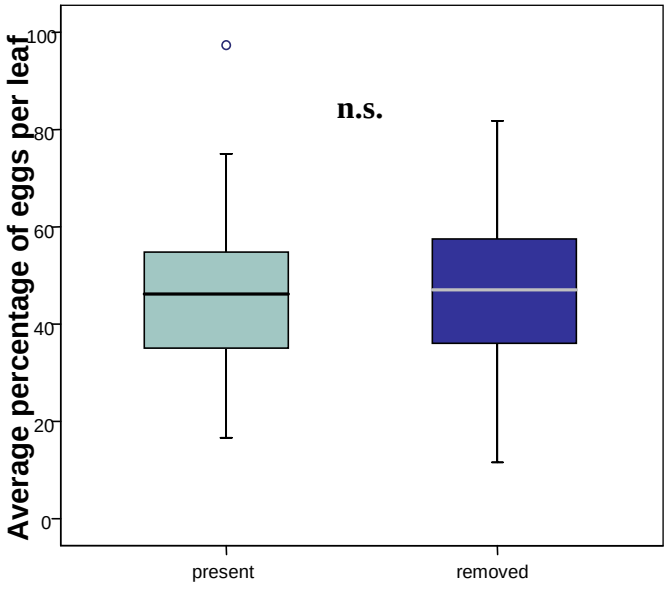
1.1 The effect of larval presence



1.1a: Effect of the surface application of phenidone on the oviposition behaviour of *P. rapae*. Larvae were removed prior to the experiment. Average number of eggs laid on Brussels sprout leaves sprayed with either phenidone or control solution. Wilcoxon Signed Ranks test Z = -0.930; p = 0.358; N = 34 . .

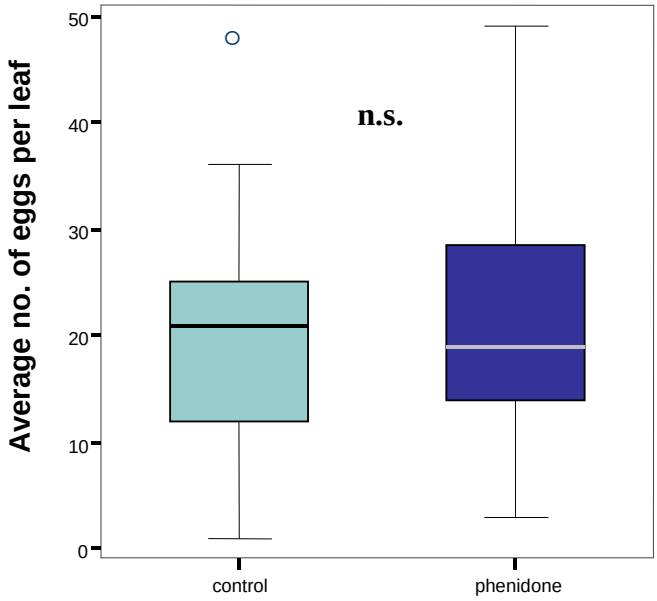


1.1b: Difference between the two surface application of phenidone experiments on which the larvae were either still present or removed prior to the experiment. Average number of eggs laid on leaves sprayed with control solution. Mann-Whitney U test Z = -0.182; p = 0.856; N = 33 and 34 respectively.

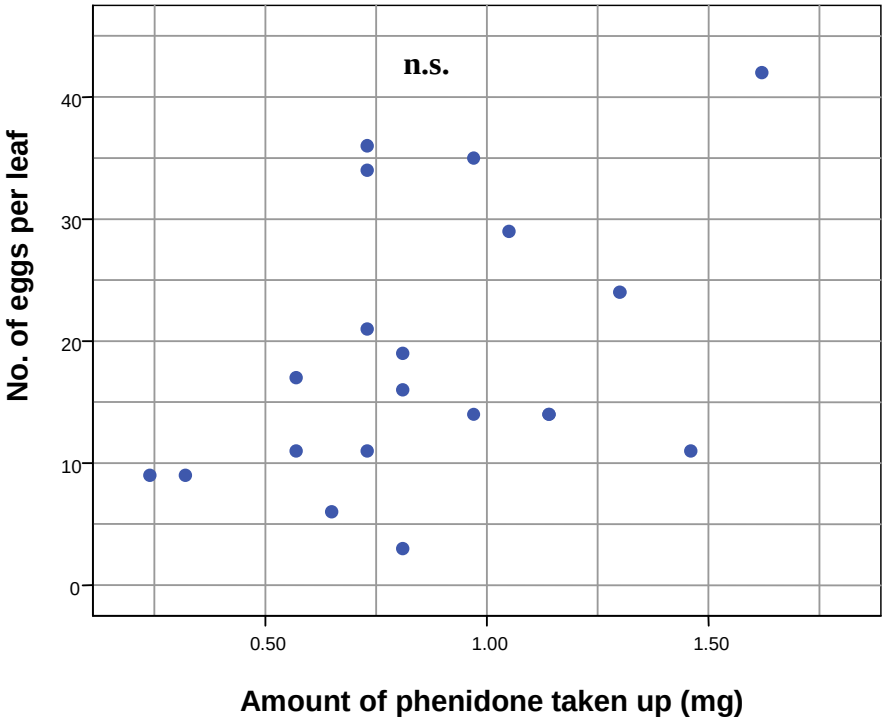


1.1c: Difference between the two surface application of phenidone experiments on which the larvae where either still present or removed prior to the experiment. Average number of eggs laid on leaves sprayed with phenidone solution. Mann-Whitney U test Z = -0.182; p = 0.856; N = 33 and 34 respectively.

1.2 Systemic uptake of phenidone



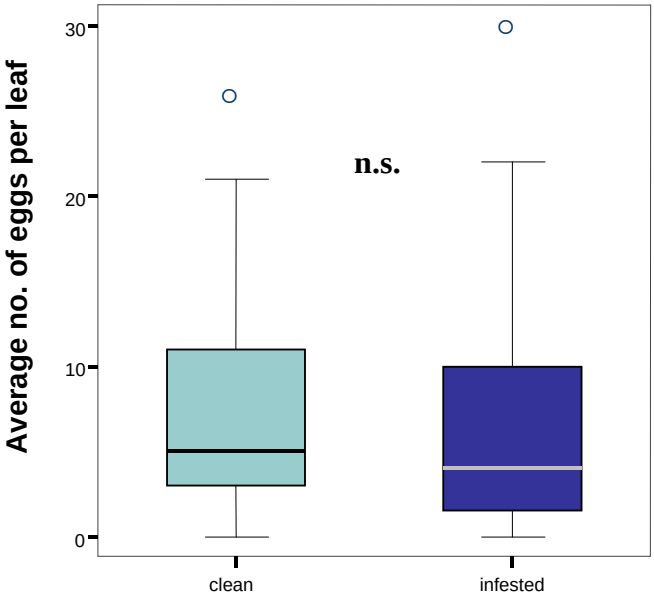
1.2a: Effect of the systemic uptake of phenidone on the oviposition behaviour of *P. rapae*. Average number of eggs laid on leaves treated with either phenidone solution or tap water. Wilcoxon Signed Ranks test Z = -0.443; p = 0.665; N = 36.



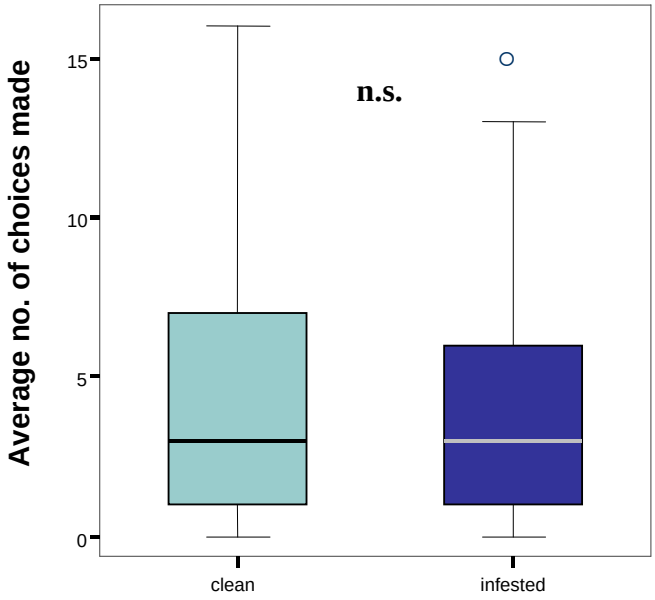
1.2b: Correlation between the amount of phenidone taken up by the leaf and the number of eggs laid on the leaf. Spearman correlation = 0.392; p = 0.086; N = 20.

Appendix 2 Effect of larval infestation experiments with *Pieris rapae*

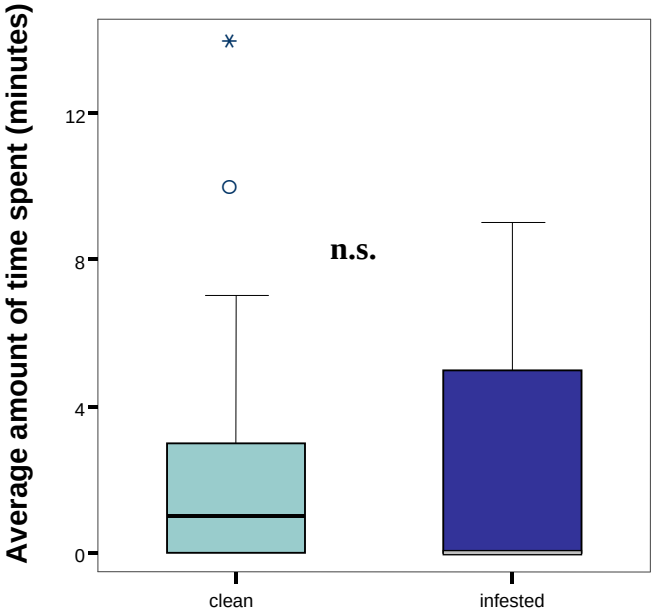
2.1 Infestation with 5 larvae per leaf



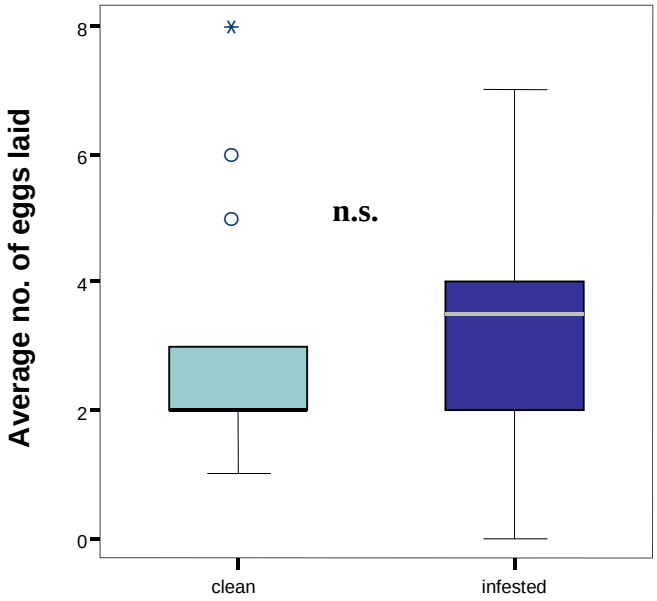
2.1a: Effect of larval infestation (5 larvae per leaf) on the oviposition preference of *P. rapae*. Average number of eggs laid within the first 15 minutes. Wilcoxon Signed Ranks test Z = -0.859; p = 0.390; N = 43.



2.1b: Effect of larval infestation (5 larvae per leaf) on the oviposition of *P. rapae*. Average number of choices made within the first 15 minutes. Wilcoxon Signed Ranks test Z = -0.694; p = 0.487; N = 43.

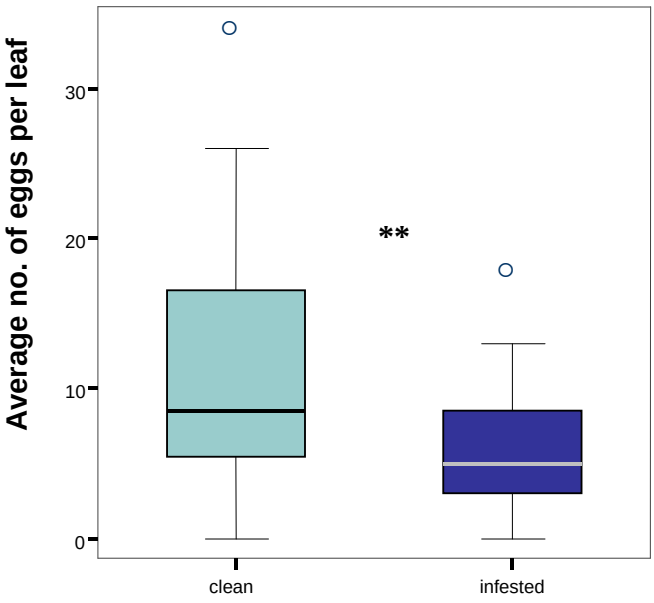


2.1c: Effect of larval infestation (5 larvae per leaf) on the oviposition preference of *P. rapae*. Average amount of time spent until the first choice was made. Mann-Whitney U test Z = -0.870; p = 0.391; N = 17 for clean, N = 22 for infested.

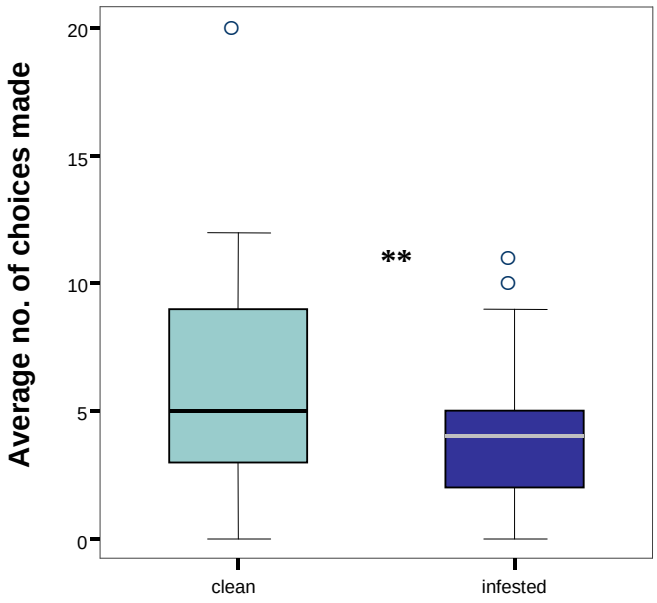


2.1d: Effect of larval infestation (5 larvae per leaf) on the oviposition preference of *P. rapae*. Average number of eggs laid during the first choice. Mann-Whitney U test Z = -0.576; p = 0.576; N = 17 for clean, N = 22 for infested.

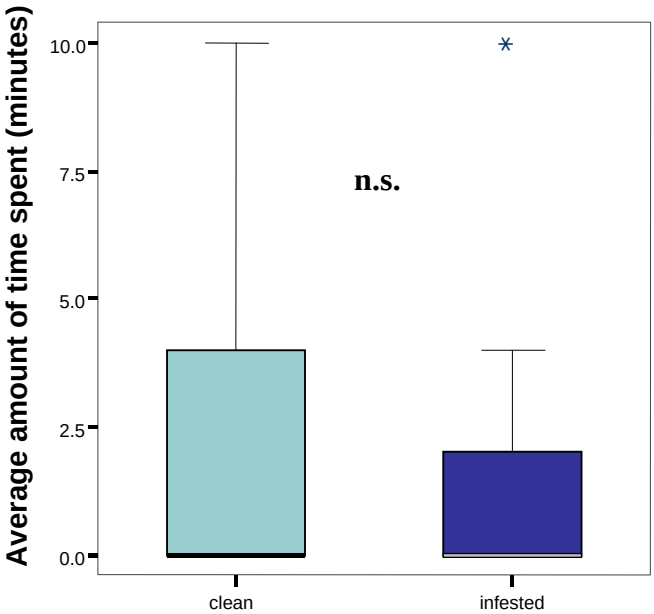
2.2 Infestation with 15 larvae per leaf



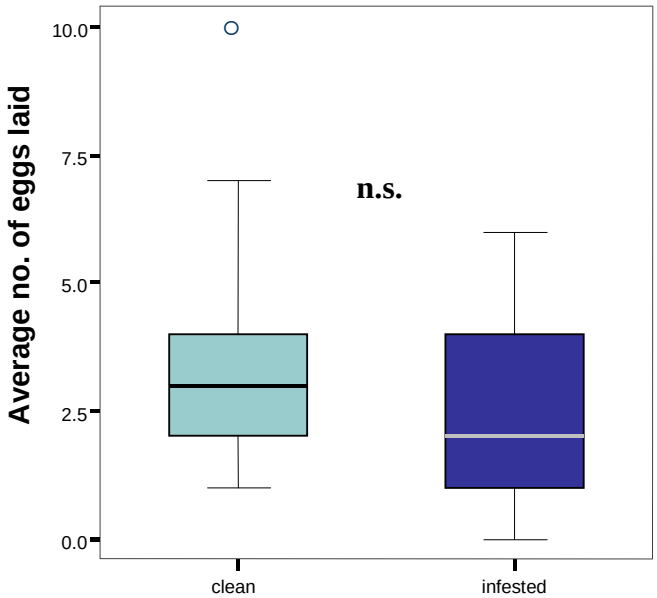
2.2a: Effect of larval infestation (15 larvae per leaf) on the oviposition preference of *P. rapae*. Average amount of eggs laid within the first 15 minutes. Wilcoxon Signed Ranks test Z = -2.930; p = 0.002; N = 24.



2.2b: Effect of larval infestation (15 larvae per leaf) on the oviposition preference of *P. rapae*. Average amount of choices made within the first 15 minutes. Wilcoxon Singed Ranks test Z = -2.611; p = 0.007; N = 24.

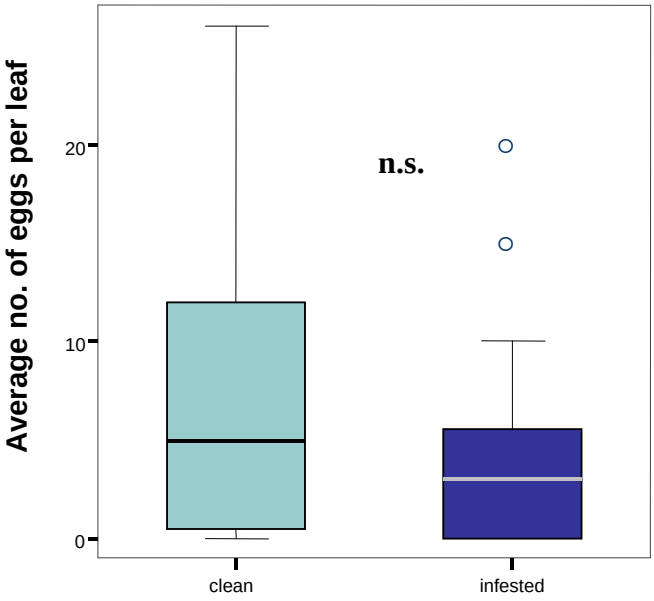


2.2c: Effect of larval infestation (15 larvae per leaf) on the oviposition preference of *P. rapae*. Average amount of time spent before the first choice was made. Mann-Whitney U test Z = -0.153; p = 0.870; N = 10 for clean, N = 13 for infested.

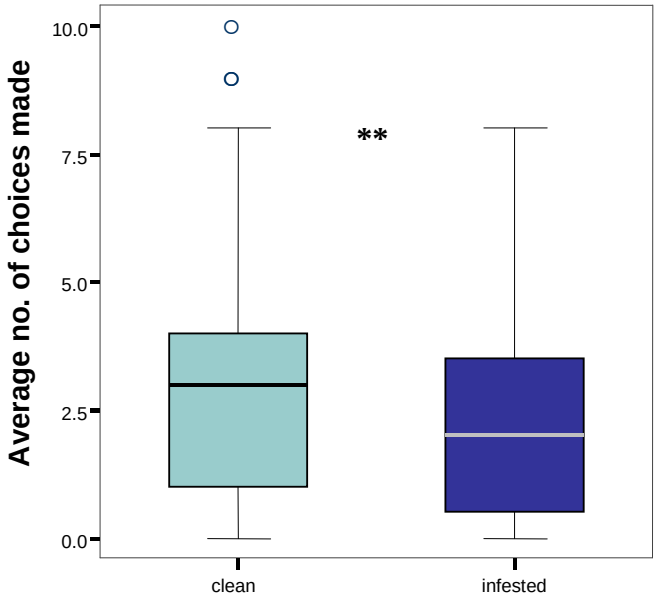


2.2d: Effect of larval infestation (15 larvae per leaf) on the oviposition preference of *P. rapae*. Average number of eggs laid during the first choice. Mann-Whitney U test Z = -1.006; p = 0.328; N = 10 for clean, N = 13 for infested.

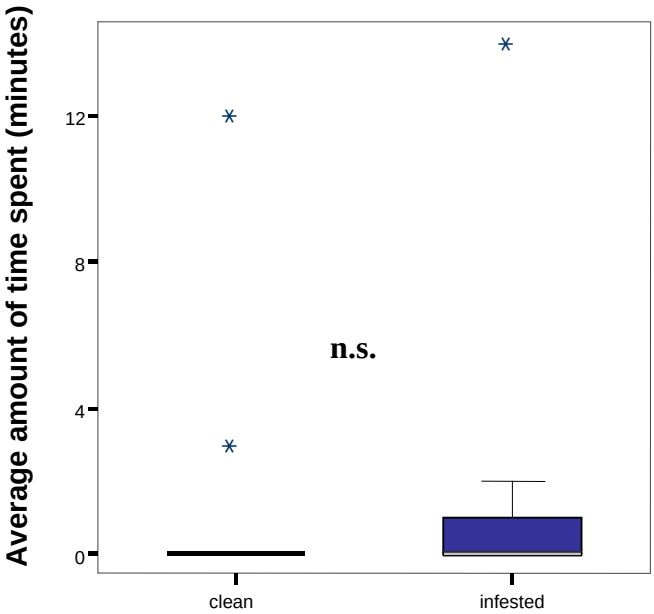
2.3 Infestation with 5 larvae per leaf, 1 week of damage



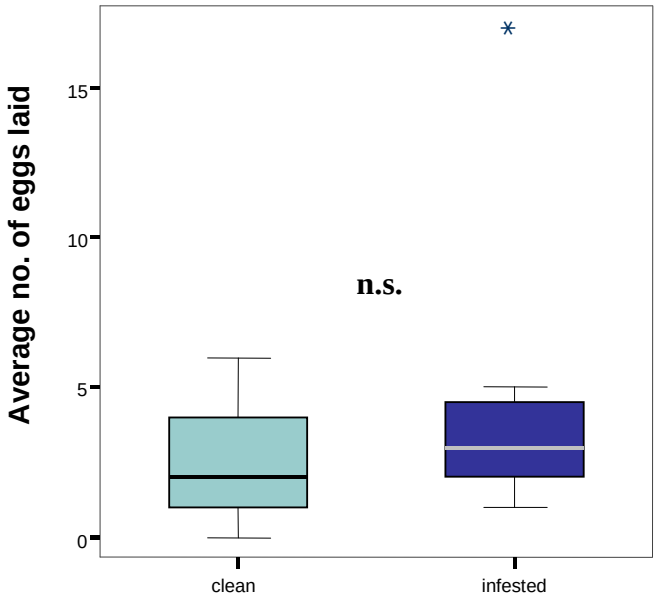
2.3a: Effect of larval infestation (1 week of damage) on the oviposition behaviour of *P. rapae*. Average number of eggs laid after the first 15 minutes. Wilcoxon Signed Ranks test Z = -1.919 p = 0.055; N = 24.



2.3b: Effect of larval infestation (1 week of damage) on the oviposition behaviour of *P. rapae*. Average number of choices made within the first 15 minutes. Wilcoxon Signed Ranks test Z = -2.578; p = 0.006; N = 24.

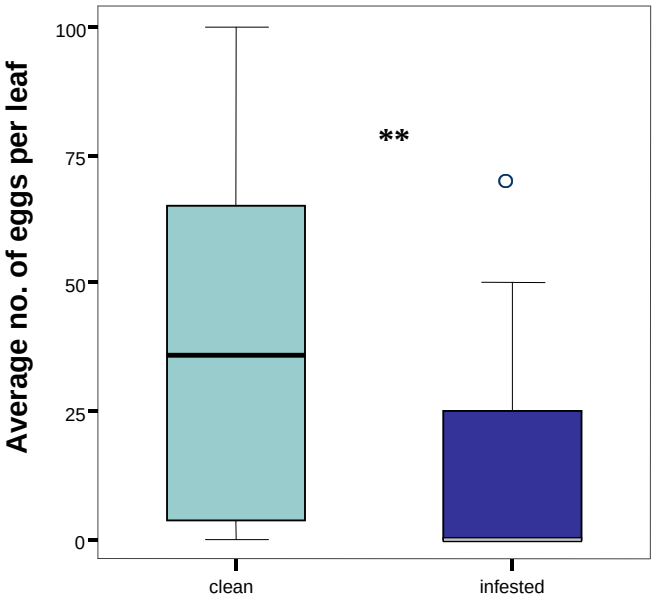


2.3c: Effect of larval infestation (1 week of damage) on the oviposition behaviour of *P. rapae*. Average amount of time spent until the first choice was made. Mann-Whitney U test Z = -0.592; p = 0.682; N = 12 replications for clean, N = 7 for infested.

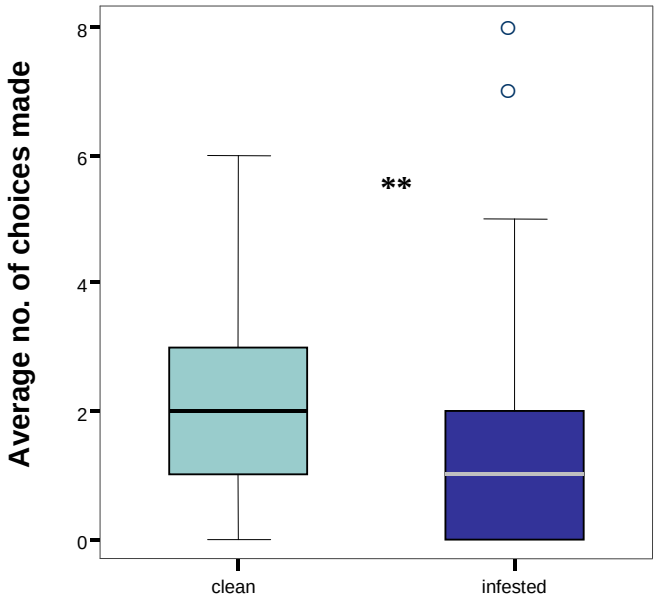


2.3d: Effect of larval infestation (1 week of damage) on the oviposition behaviour of *P. rapae*. Average number of eggs laid during the first choice. Mann-Whitney U test Z = -0.859; p = 0.422; N = 12 for clean, N = 7 for infested.

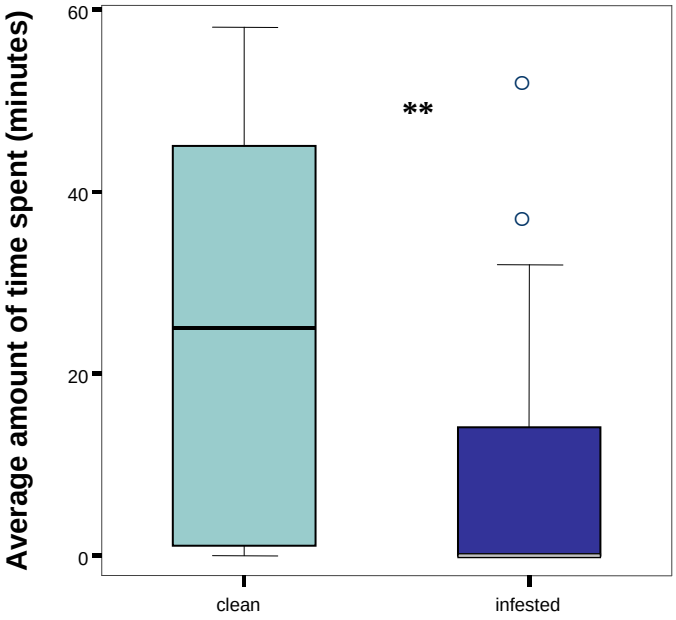
Appendix 3 Effect of larval infestation experiments with *Pieris brassicae*



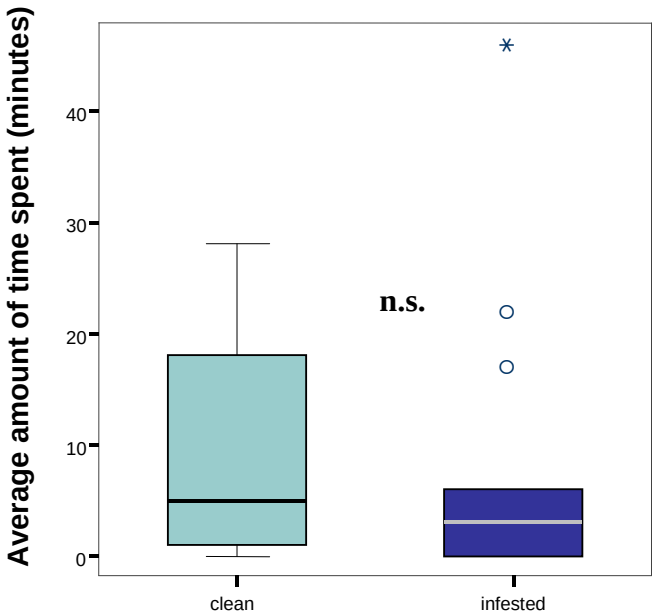
3a: Effect of larval infestation (5 larvae per leaf) on the oviposition preference of *P. brassicae*. Average number of eggs laid during the first 60 minutes (estimate). Wilcoxon Signed Ranks test Z = -2.656; p = 0.007; N = 31.



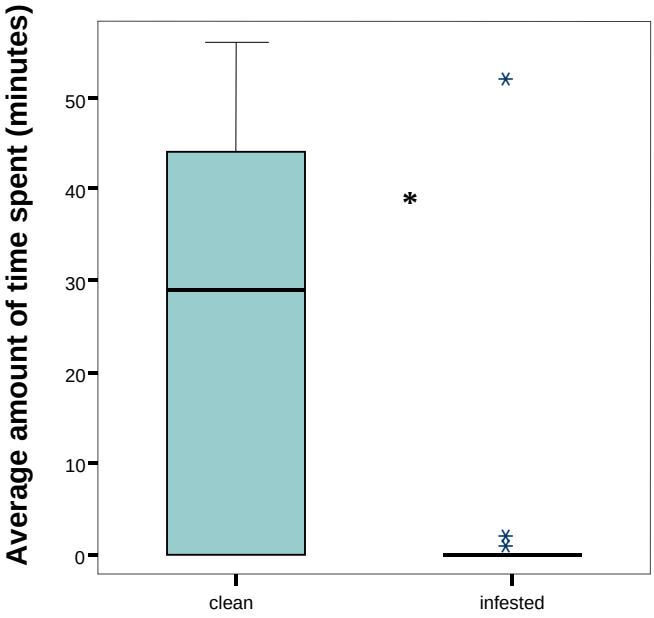
3b: Effect of larval infestation (5 larvae per leaf) on the oviposition preference of *P. brassicae*. Average number of choices made during the first 60 minutes. Wilcoxon Signed Ranks test Z = -2.866; p = 0.004; N = 31.



3c: Effect of larval infestation (5 larvae per leaf) on the oviposition preference of *P. brassicae*. Average amount of time spent on the leaf within the first 60 minutes. Wilcoxon Signed Ranks test Z = -2.844; p = 0.003; N = 31.



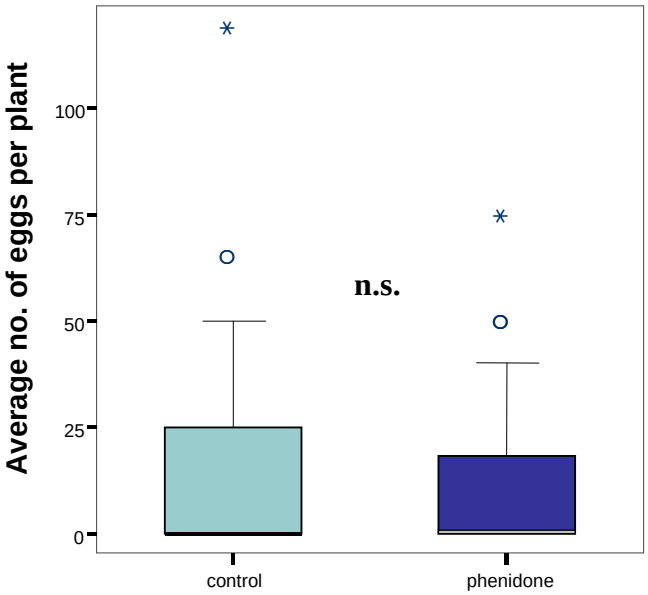
3d: Effect of larval infestation (5 larvae per leaf) on the oviposition preference of *P. brassicae*. Average amount of time spent until the first choice was made. Mann-Whitney U test Z = -0.826; p = 0.421; N = 15 for clean, N = 13 for infested.



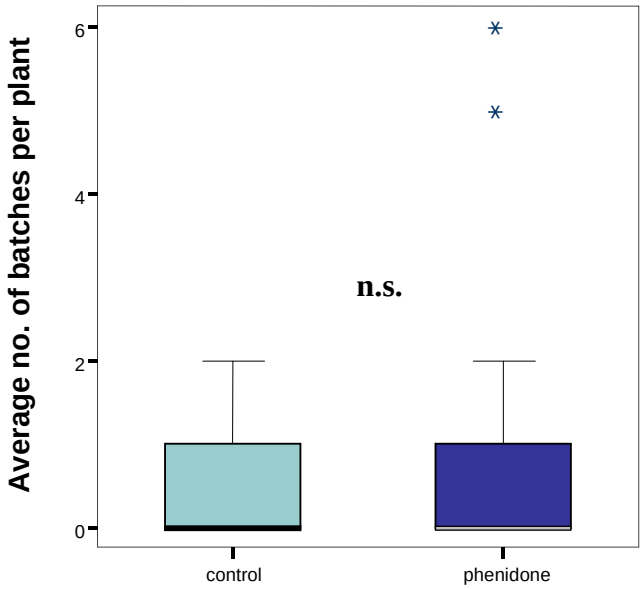
3e: Effect of larval infestation (5 larvae per leaf) on the oviposition preference of *P. brassicae*. Average amount of time spent on the leaf during the first choice. Mann-Whitney U test Z = -2.428; p = 0.014; N = 15 for clean, N = 13 for infested.

Appendix 4 Effect of phenidone experiments with *Pieris brassicae.*

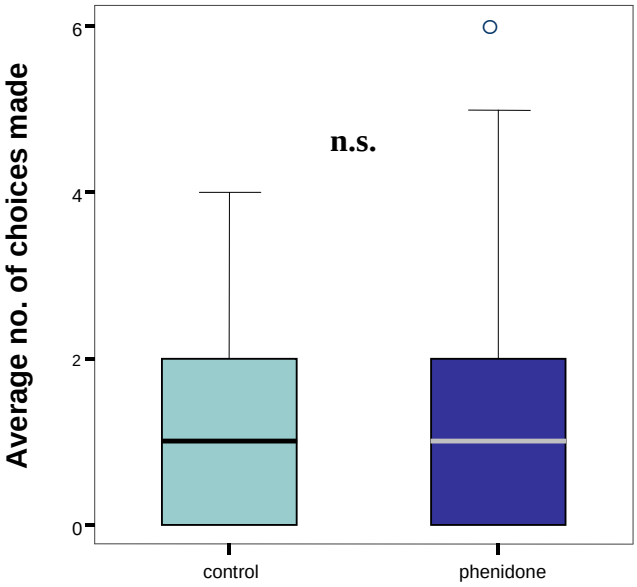
4.1 The effect of pure phenidone



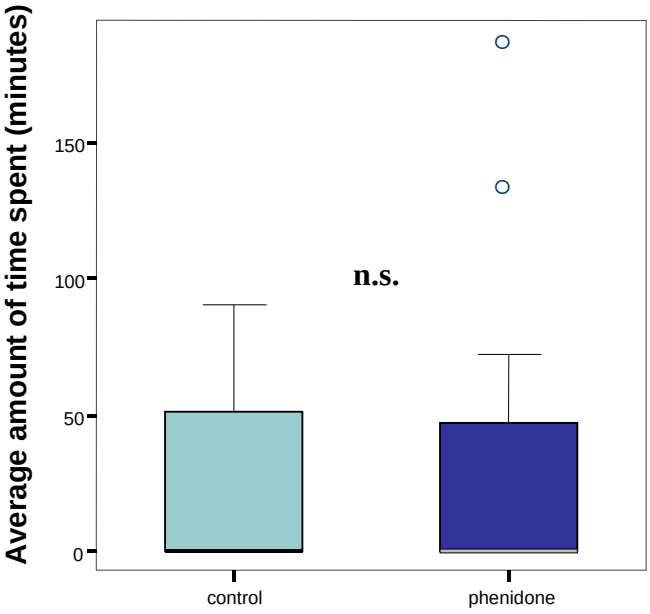
4.1a: Effect of pure phenidone on the oviposition behaviour of *P. brassicae*. Average number of eggs laid within the first 60 minutes (estimate). Wilcoxon Signed Ranks test Z = -0.020; p = 0.992; N = 22.



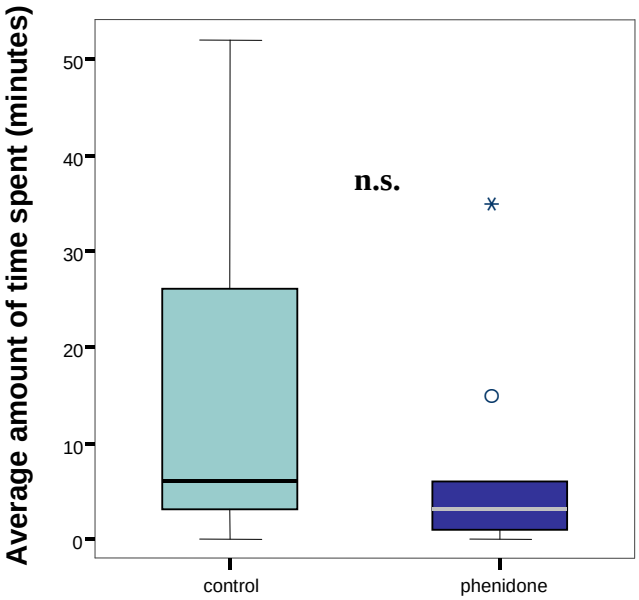
4.2b: Effect of pure phenidone on the oviposition behaviour of *P. brassicae*. Average number of batches found after the first 60 minutes. Wilcoxon Signed Ranks test Z = -0.615; p = 0.563; N = 22.



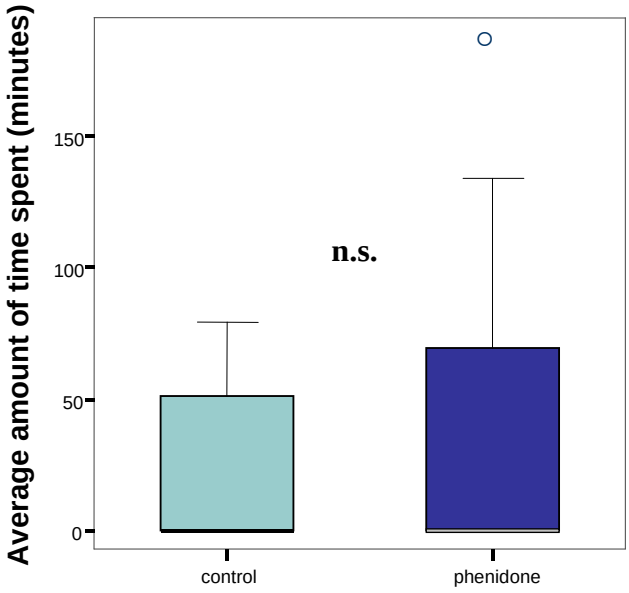
4.1c: Effect of pure phenidone on the oviposition behaviour of *P. brassicae*. Average number of choices made within the first 60 minutes. Wilcoxon Signed Ranks test Z = -0.789; p = 0.798; N = 22.



4.1d: Effect of pure phenidone on the oviposition behaviour of *P. brassicae*. Average amount of time spent on the plant within the first 60 minutes. Wilcoxon Signed Ranks test Z = -0.044; p = 0.974; N = 22.

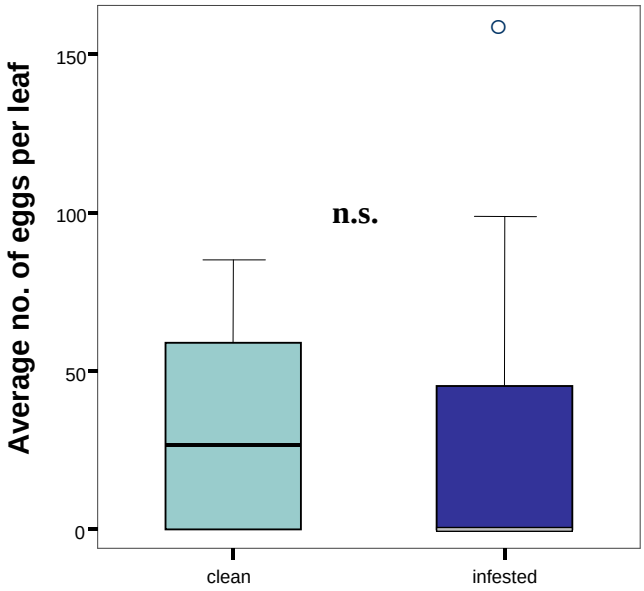


4.1e: Effect of pure phenidone on the oviposition behaviour of *P. brassicae*. Average amount of time spent until the first choice was made. Mann-Whitney U test Z = -0.890; p = 0.395; N = 9 for control, N = 9 for phenidone.

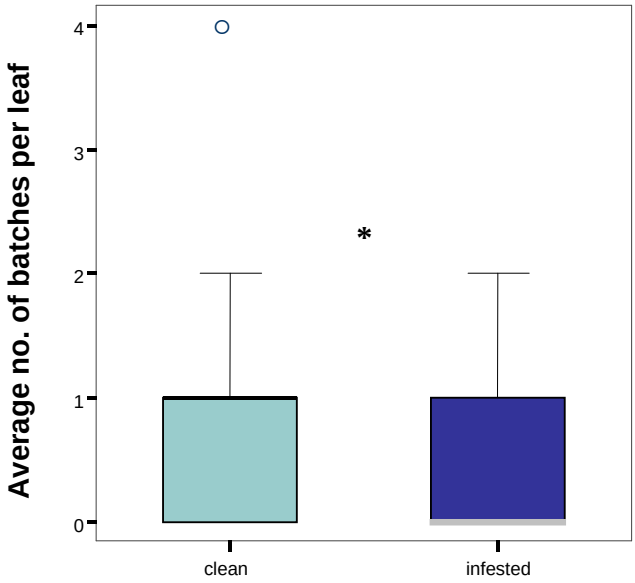


4.1f: Effect of pure phenidone on the oviposition behaviour of *P. brassicae*. Average amount of time spent on the plant during the first choice. Mann-Whitney U test Z = -0.338; p = 0.698; N = 9 for control, N 9 for phenidone.

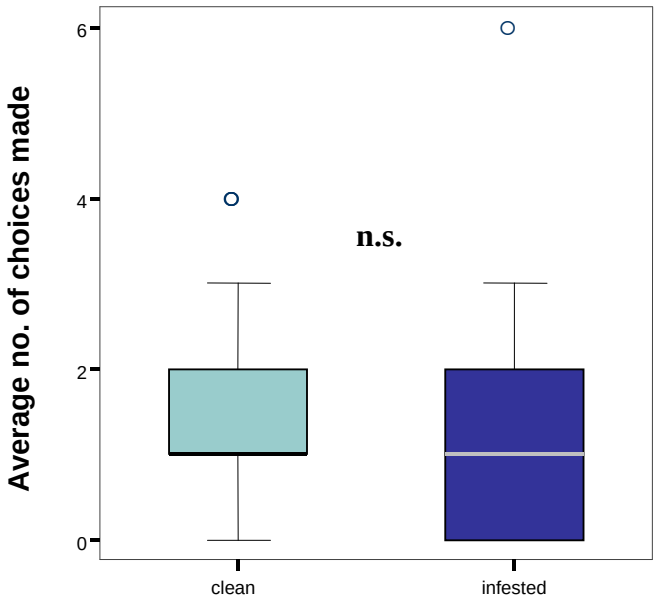
4.2 The effect of larval infestation with phenidone-sprayed plants



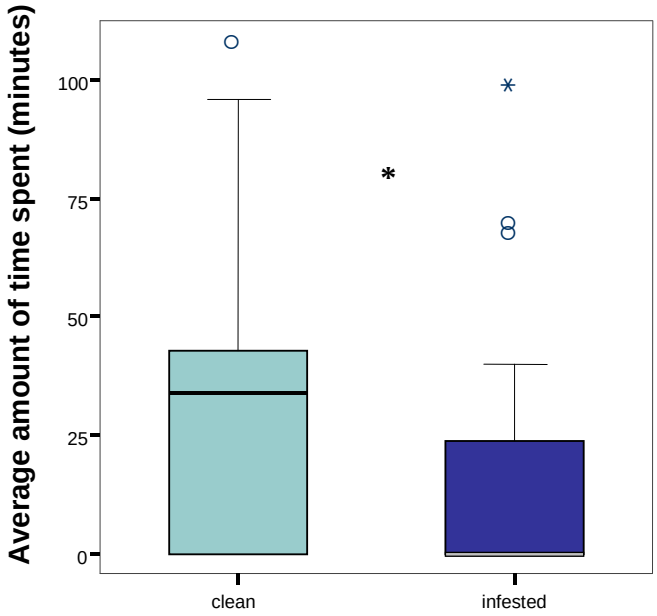
4.2a: Effect of larval infestation (5 larvae per leaf) with phenidone-sprayed plants on the oviposition behaviour of *P. brassicae*. Average number of eggs laid within the first 60 minutes (estimate). Wilcoxon Signed Ranks test Z = -1.833; p = 0.060; N = 33



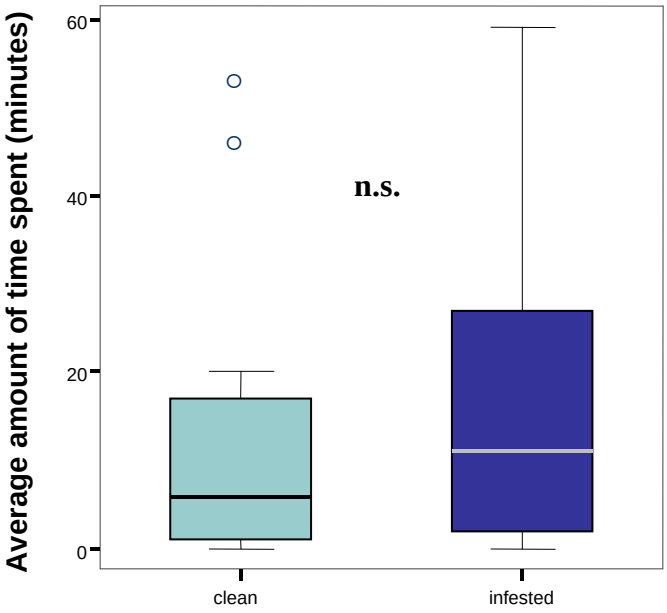
4.2b: Effect of larval infestation (5 larvae per leaf) with phenidone-sprayed plants on the oviposition behaviour of *P. brassicae*. Average number of batches found after the first 60 minutes. Wilcoxon Signed Ranks test Z = -2.527; p = 0.015; N = 33.



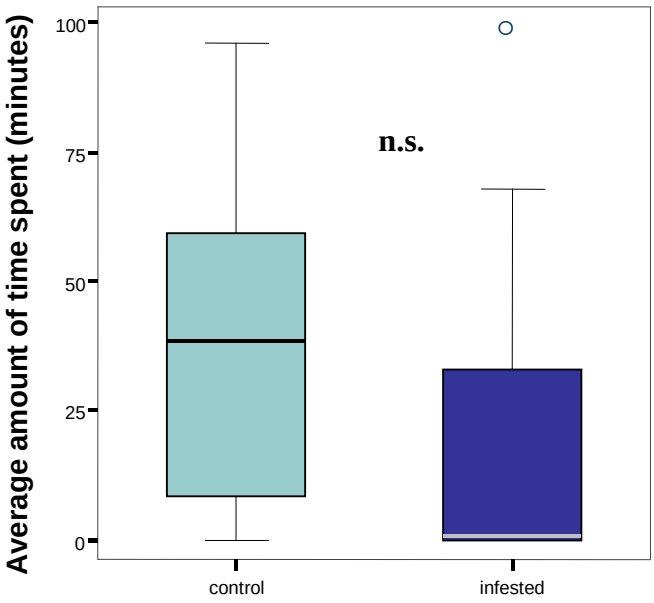
4.2c: Effect of larval infestation (5 larvae per leaf) with phenidone-sprayed plants on the oviposition behaviour of *P. brassicae*. Average number of choices made within the first 60 minutes. Wilcoxon Signed Ranks test Z = -1.548; p = 0.131; N = 33



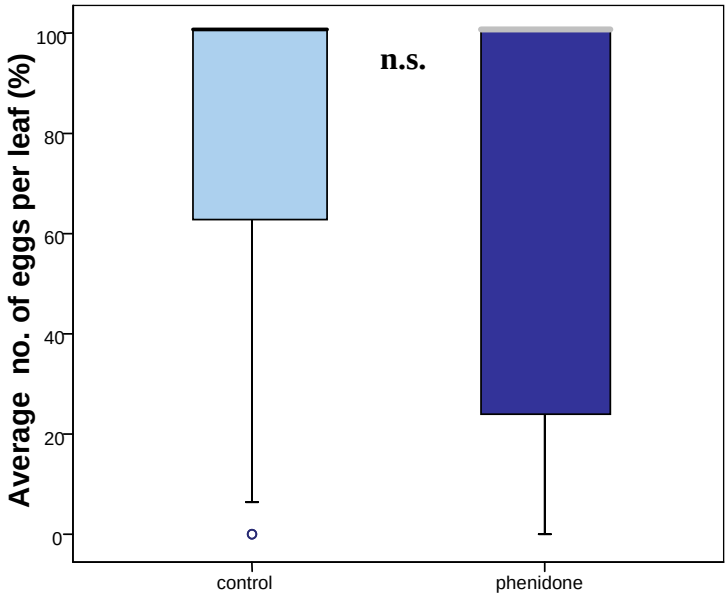
4.2d: Effect of larval infestation (5 larvae per leaf) with phenidone-sprayed plants on the oviposition behaviour of *P. brassicae*. Average amount of time spent on the leave within the first 60 minutes. Wilcoxon Signed Ranks test Z = -2.108; p = 0.034; N = 33.



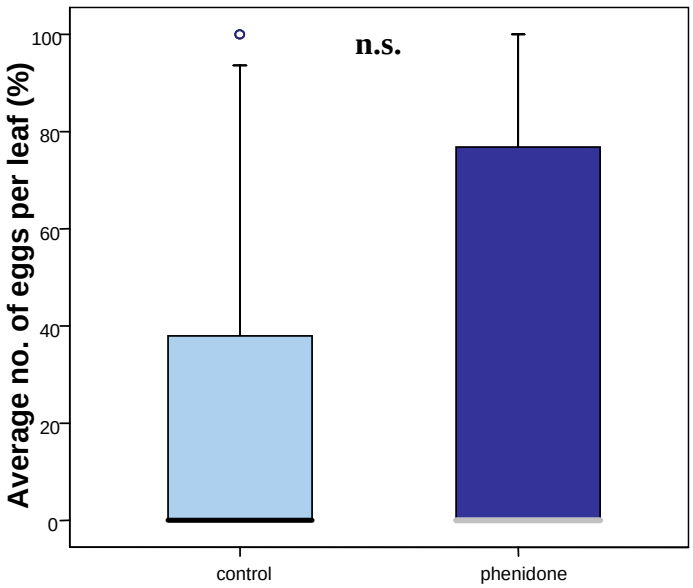
4.2e: Effect of larval infestation (5 larvae per leaf) with phenidone-sprayed plants on the oviposition behaviour of *P. brassicae*. Average amount of time spent until the first choice was made. Mann-Whitney U test Z = -0.438; p = 0.673; N = 16 for clean, N = 14 for infested.



4.2f: Effect of larval infestation (5 larvae per leaf) with phenidone-sprayed plants on the oviposition behaviour of *P. brassicae*. Average amount of time spent on the leaf during the first choice. Mann-Whitney U test Z = -1.821; p = 0.070; N = 16 for clean, N = 14 for infested.

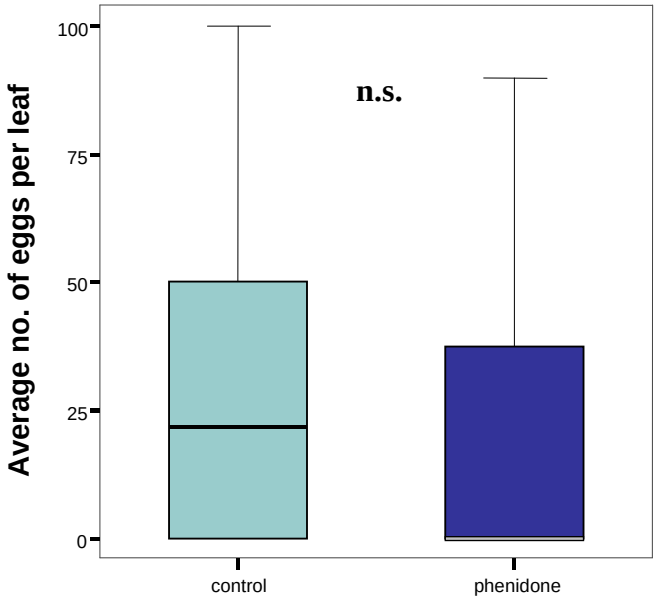


4.2g: Difference between the two effect of larval infestation experiments using either control or phenidone-sprayed plants. Average total amount of eggs laid on clean plants. Mann-Whitney U test Z = -0.060; p = 0.952; N = 31 for control, N = 33 for phenidone.

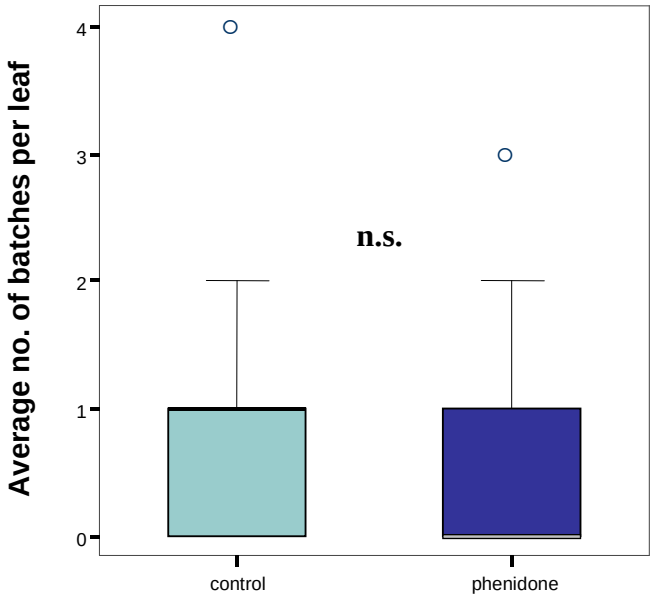


4.2h: Difference between the two effect of larval infestation experiments using either control or phenidone-sprayed plants. Average total amount of eggs laid on infested plants. Mann-Whitney U test Z = -0.060; p = 0.952; N = 31 for control, N = 33 for phenidone.

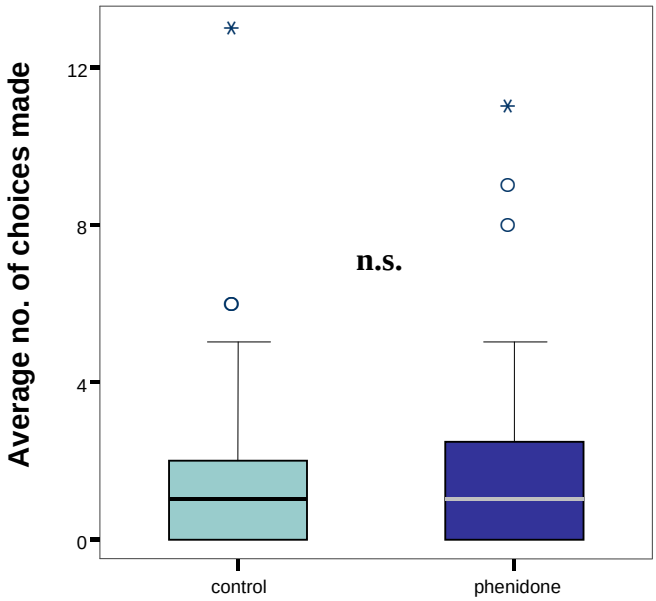
4.3 Effect of the surface application of phenidone with infested plants



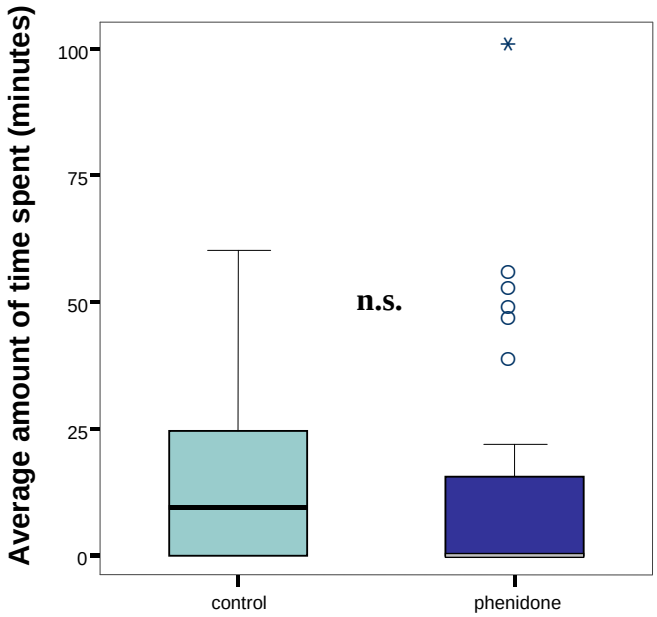
4.3a: Effect of the surface application of phenidone on the oviposition behaviour of *P. brassicae*. Average number of eggs laid within the first 60 minutes (estimate). Wilcoxon Signed Ranks test Z = -1.272; p = 0.209; N = 36



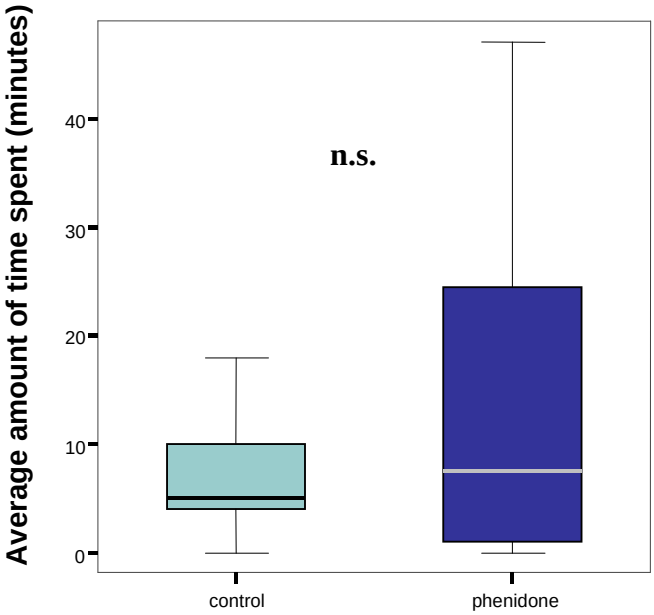
4.3b: Effect of the surface application of phenidone on the oviposition behaviour of *P. brassicae*. Average number of batches found after the first 60 minutes. Wilcoxon Signed Ranks test Z = -1.502; p = 0.370; N = 36.



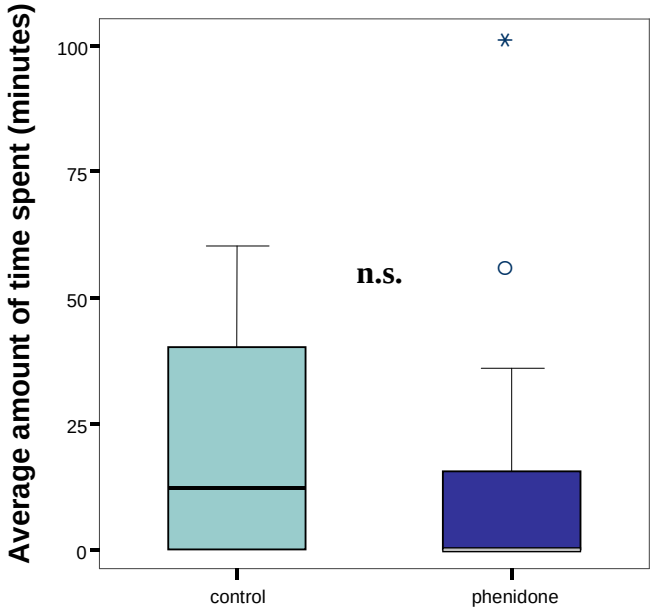
4.3c: Effect of the surface application of phenidone on the oviposition behaviour of *P. brassicae*. Average number of choices made within the first 60 minutes. Wilcoxon Signed Ranks test Z = -0.307; p = 0.770; N = 36.



4.3d: Effect of the surface application of phenidone on the oviposition behaviour of *P. brassicae*. Average amount of time spent on the leaf during the first 60 minutes. Wilcoxon Signed Ranks test Z = -0.915; p = 0.370; N = 36.



4.3e: Effect of the surface application of phenidone on the oviposition preference of *P. brassicae*. Average amount of time spent until the first choice was made. Mann-Whitney U test Z = -0.793; p = 0.440; N = 13 for control, N = 16 for phenidone.



4.3f: Effect of the surface application of phenidone on the oviposition preference of *P. brassicae*. Average amount of time spent on the leaf during the first choice. Mann-Whitney U test Z = -1.134; p = 0.271; N = 13 for control, N = 16 for phenidone.