Egg laying behaviour of the large pine weevil, *Hylobius abietis*

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The institute SLU

SLU (Sveriges lantbruksuniversitet/Swedish University of Agricultural Sciences) in Uppsala is an agricultural university. Uppsala is one of the 4 main campuses. In total there are 27 locations where education and research is carried out. Around 3200 people are employed at the university, spread over the different locations.

The university is divided in 4 different faculties:

- Faculty of Landscape Planning, Horticulture and Agricultural Science
- Faculty of Natural Resources and Agricultural Sciences
- Faculty of Veterinary Medicine and Animal Science
- Faculty of Forest Sciences.

SLU's objective is: 'to take responsibility for the development of learning and expertise in areas concerning biological resources and biological production.'

The Department of Entomology, where I did my Internship, resorts under the Faculty of Natural Resources and Agricultural Sciences. This department carries out research on different aspects of insect biology; mainly population ecology, chemical ecology, ethology and genetics. Teaching is given at all levels of the programs in agronomy, natural resources and forestry.

Around 50 people work in this department, from which ~ 25 researchers/(assoc.) professors, 10 PhD students, 15 people as technical and administrative staff, and on average 5 MSc students.

One aim from the department is to generate knowledge that can improve plant protection in forestry and agriculture. This research deals with the variability of insect population densities in space and time, the behaviour of insects and the importance of chemical signals for insects and plants. Another research field is nature conservation, which focuses on habitat requirements and

dispersal biology of threatened species, and the spatial distribution and population genetics of insects in forests and agricultural landscapes. There is also research done which is related to beekeeping, focusing on pathology of honey bees and bumble bees.

The research at the Entomology department can be separated into 9 different areas:

- Conservation biology
- Honey bee pathology and Apiculture
- Insect physiology
- Medical entomology
- Molecular ecology
- Pest management
- Plant-insect interactions
- Population dynamics
- Taxonomy

My project was part of the pest management area. This area focuses on the insect species that cause economically important damage in forestry and agriculture. The aim of the research is to develop methods to predict and reduce damage levels and to find methods which are environmentally friendly and sustainable, through which the amount of insecticides can be reduced. Important research areas include behaviour-modifying chemicals, natural enemies of pest insects, plant resistance against herbivores, and monitoring of pest species.

The project I worked on was part of the *Hylobius* program. This research program aims to provide methods to reduce damage caused by the pine weevil, *Hylobius abietis*, to an acceptable level without use of insecticides.

1. Introduction

Figure 1: An adult *Hylobius abietis*.

The pine weevil, *Hylobius abietis* (Linnaeus) (Coleoptera: Curculionidae) is an important pest species in forests in western and northern Europe. The adult weevils feed on tender bark of conifer seedlings and on the crowns and the roots of mature coniferous trees (Örlander et al., 2000). The feeding on the stem bark of the young seedlings causes serious problems for the forest regeneration programmes in Nordic countries (Day et al., 2004).

The weevil breeds in conifer stumps and feeds extensively on conifer seedlings, so it is especially a problem in countries where clear-cut plantation forestry is employed (Day et al., 2004). During feeding, the phloem is damaged and the bark is removed, which kills the seedlings. Damage by the pine weevil frequently cause girdling of seedlings but thicker stems have a lower risk of being girdled than thinner stems (Thorsén et al., 2001).

During the oviposition period, female weevils can consume up to 50% more bark than males do. When the oviposition ceases, the feeding rate declines to the same level as for males (Bylund et al., 2004).

The periods with the highest risk of damage are: May-August on fresh clear cuttings, late July-September on 1-year-old clear cuttings and April-May on 2-year-old clear cuttings (Nordlander, 1987; Örlander et al., 1997).

1.1 Life cycle of Hylobius abietis

The length of the life cycle of *H. abietis* is dependent on different factors. In warmer climates, the developmental time can be 2 years, while in cooler climates, the time to develop from egg till adult can take 3 years. Another factor

influencing the length of the life cycle is the time when the eggs are deposited (early or late in the summer) (Elton, 1962). In this report we describe the life cycle of 2 years (Figure 2), because this is life cycle of the weevils used in this study.

The oviposition starts in June and lasts till early August (Nordenhem, 1989). The eggs are laid in stumps of conifer trees as well as in the soil surrounding host material. More details about the egg laying will be described in § 1.2. The eggs hatch after 2-3 weeks and the larvae feed by making a feeding tunnel in the bark (in the cambial region) of roots of recently killed or dying conifer trees and in fresh stumps. When the eggs are deposited in the soil, the larvae migrate to a suitable feeding site in the bark (Nordenhem & Nordlander, 1994; Nordlander et al., 1997). They stay in the stumps and eat until they are full-grown. At the end of their feeding tunnel, they make a pupal chamber and then a period of rest begins. They stay in their pupal chamber during this period. This over wintering stage starts before the winter and lasts till June-July of the next year. After this rest period they pupate and after 2-3 weeks the adult weevils emerge (Elton, 1962). Some newly emerged weevils leave the pupal chamber in July-September the same year and over winter in the ground after a period of feeding. Other weevils stay in their pupal chambers and do not emerge above the ground until spring of the third year (Nordenhem, 1989).

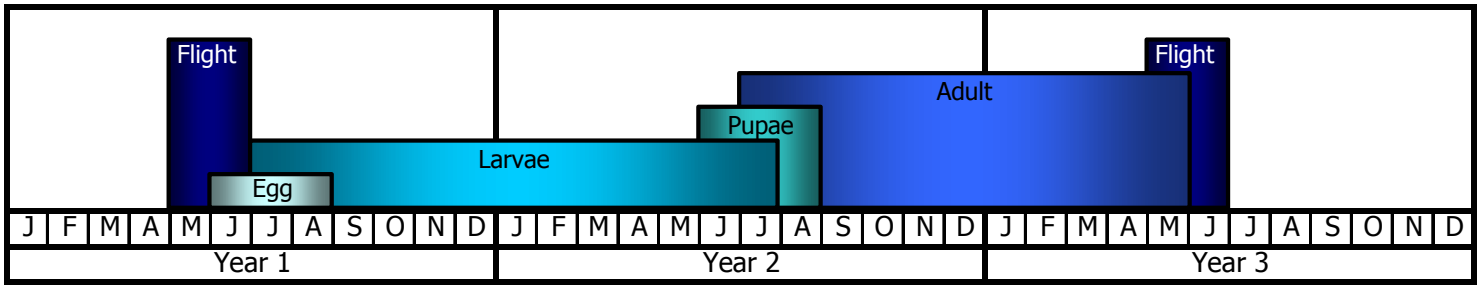

Figure 2: The 2-year life cycle of *Hylobius abietis*

In the beginning of May, the adult weevils start to develop their flight muscles to migrate to new breeding areas. The actual flight period is from May till June.

One individual is capable of covering distances up to 80 km, but most of the population will fly around 1.5 km during their flight period (Solbreck, 1980). The flying weevils are attracted to host volatiles emanating from stumps and logging waste. When a new breeding place is found, the weevils start feeding on the trees for a short period of time. During this period the females become sexually mature and the oviposition starts (Örlander et al., 2000).

1.2 Egg laying and protection of eggs

Female pine weevils chew niches in the inner bark of roots of freshly killed trees and deposit their eggs into these niches. One egg is laid at the time and they can be laid singly or in irregular groups. The choice of the egg-laying site is dependent on factors such as the soil moisture and soil texture (Bylund et al. 2004).

For a long time it was thought that eggs were only laid in the bark, but Nordlander et al. (1997) found that under natural field conditions the eggs are mostly deposited in the soil surrounding the host material. Most of the larvae migrated from the soil to the nearby host material. Two explanations given by Nordlander et al. (1997) for the laying of the eggs in the soil instead of the bark are:

1. The egg predation by other arthropods or by conspecifics is avoided. Some predators live in the bark surface of the roots and are strongly attracted to a combination of host monoterpenes and ethanol. This is the same combination of odours, which *H. abietis* uses to locate suitable breeding material, so there is a possibility that these odours are present at the location of the eggs.

   Destruction of conspecific eggs by *H. abietis* can be intentional or unintentional. Experiments show that weevils can feed extensively next to places where eggs are laid, however in the field this behaviour has not been

observed. Therefore it is not very plausible that such cannibalism influences the egg laying behaviour, but it still is a possibility.

2. The larvae are better in choosing suitable feeding sites than the female weevils. The larvae emerge 4-5 weeks after the eggs are laid. During this period the quality of the root bark can change. In this way the larvae may be more capable of finding a suitable host material than the female weevil. Moreover, other larvae may have already started feeding on the roots, thereby affecting the choice of the newly hatched larvae.

It is known from other insect species that various protective agents can be added to the egg in order to avoid predation or to deter conspecific females from laying their eggs there. This protection can be in the form of faeces, toxic hairs covering the eggs, a sticky substance in which (parts of) natural enemies get stuck. Also defensive compounds can be added within the egg which can act as a repellent, feeding deterrents or a respiratory inhibitor (Blum & Hilker, 2002).

The Cabbage seed weevil, *Ceutorhynchus assimilis*, has an uniform egg dispersion in the field. This uniformity is caused by an oviposition deterring pheromone added on the winter rape pod, in which the female weevil lays her eggs. After the egg laying, the female brushes the pod, meanwhile adding the deterring pheromone. This pheromone deters other females from oviposition in the same host, and thus results in larval resource partitioning (Kozlowski et al. 1983). In this way the fitness of the weevil is increased, since the competition for a restricted food source is reduced or eliminated (Ferguson & Williams, 1991). Ferguson et al (1999) concluded that this pheromone is secreted by the epidermal cells of the eighth abdominal tergite.

1.3 Research objectives

Methods used to control *H. abietis* are for example the treatment of seedlings with an insecticide (e.g. permethrin) and soil scarification. However this use of insecticides may become forbidden in Sweden due to environmental reasons. Because soil scarification alone is not sufficient (Thorsén et al. 2001), other (biological) control methods need to be investigated. Since there is not much known about the egg laying behaviour of *H. abietis*, this was chosen as main topic for this study.

The objectives of this study are:

- To see whether the placing of an egg by a female weevil depends on where another female weevil previously had laid her eggs.
- To investigate if the distribution of eggs laid by a *H. abietis* female is affected by previous egg laying by simultaneous exposure to bark with and without eggs and feeding scars.
- To determine if chemicals present in the eggs deter *H. abietis* females from feeding.
- To determine if chemicals present in the faeces deter *H. abietis* females from feeding.
- To compare the response of *H. abietis* when exposed to 'clean' eggs, which had been laid in an empty Petri dish, vs. eggs contaminated with faeces that had been laid in bark.
- To find a good experimental set-up to test the objectives mentioned above.
- To describe the behavioural steps of the egg laying in *H. abietis*

To accomplish these objectives, different experimental set-ups were developed. The set-ups were adjusted over time, to find a convenient set-up which gives the relevant information. The egg laying behaviour was studied by observations through a stereo-microscope.

2. Materials

The adult pine weevils used in the experiments were collected at sawmills during their flight period in June 2004 at two locations in southern and central Sweden: Asa (province of Småland) and Svärdsjo (province of Dalarna). Three different groups of weevils were used: one from the Svärdsjö location at 2 June 2004 and two from the Asa location at 28 June 2004 (called Asa I and Asa II).

The Scots pine bark used for the experiments was collected from trees from different locations around Uppsala. Stem pieces were used for feeding the weevils, while the smaller branches were used for the experiments.

The weevils were stored in groups of around 200-300 individuals in wooden boxes. The wooden boxes were placed in a dark room kept at 10 °C in order to interrupt the weevils' reproductive development. Two weeks before the experiments were started, weevils were transferred from the 10 °C room to the laboratory (20 °C, LD 8:16). In these two weeks the weevils became reproductive and were ready for the experiments. The weevils in the laboratory were kept in buckets with branches of pine added. Water was supplied in a glass tube closed with a piece of soft paper. The food and water were renewed every week. According to previous experience the weevils can be used in experiments for approximately two months. However, in these experiments severe problems with the conditions of the weevils were observed, which will be described in § 2.1. Due to these problems new groups of weevils were transferred from the 10 °C room to the laboratory.

Methods used for the various experiments are described in Chapter 3. The experiments were performed in the laboratory (20 °C, LD 18:6).

2.1 Gregarines

2.1.1 Gregarines: Observations

Problems with the condition of the weevils occurred during the experiments. In October 2004 it was observed that the egg laying of the females had not started yet. The group (which was collected at the Svärdsjö location) had been transferred from the 10°C storage approximately three weeks before. Some weevils were dissected to observe the ovaries of the females. During the dissection, a considerable amount of gregarines (Protozoa: Eugregarinidae) were found in the midgut and gut (Figure 3). More about the biology of the gregarines is described in § 3.1.2.

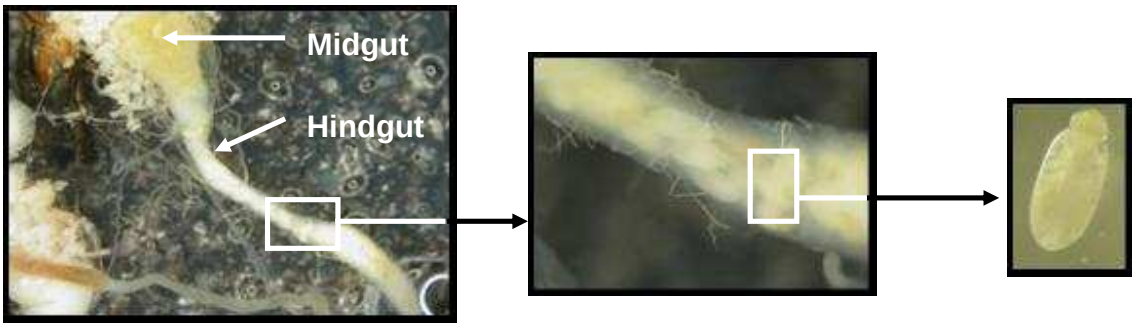

Figure 3: Dissection of a *Hylobius abietis* female. On the left picture, part of the midgut and hindgut, containing gregarines is shown. The picture in the middle shows the gut with gregarines and the picture on the right shows one single (mature) gamont. Photo's: M. Munneke

The weevils kept in the 10 °C room were also checked for gregarines. From each wooden box, five weevils were collected. There were three boxes from Asa. From the first box, five weevils contained gregarines. From second box, one of the weevils contained gregarines and from the last box, two contained gregarines. In the box from Svärdsjö, all of five sampled weevils had gregarines, as well as the weevils that had been transferred from the 10 °C room to the laboratory in September (also collected at Svärdsjö). In general, the weevils collected from the Svärdsjö location were more infected than the weevils from the Asa collection. Possibly, the Asa population was not infected at the time of collection but

contamination could have occurred during the storage in the 10 °C room, since weevils escaped sometimes from the wooden boxes and were put back randomly in a box.

The density of weevils was high both during storage (10 °C) and in the laboratory. The food was changed once a week in the laboratory and once per two months in the storage. Because of these two factors, it is possible that the food contained a high amount of gregarines spread by the faeces of infected weevils and that a considerable part of the populations were infected.

During the dissection of the weevils, it was difficult to distinguish the different parts of the gut because the midgut seemed more or less destroyed.

Weevils used for other feeding tests were also dissected. Six weevils that did not feed during the experiment and five weevils which fed a lot during the experiment were dissected.

The weevils that had fed much all contained gregarines, but the gut was full of faeces and the gregarines surrounded the faeces. The midgut was intact in four of the five weevils. Gregarines were also found in the midgut (in low amounts) but were not found in the hindgut. In one weevil the midgut contained gregarines that were attached to each other, with the upper side of the first connected to the under side of the second (association of two mature gamonts). The gregarines were situated only in the upper part of the gut, which could be an indication that they are spread from the midgut.

The weevils that had not fed had hardly any faeces in their gut. The midgut could often not be distinguished or it was very small or looked ruptured. All five weevils contained gregarines, but not in large amounts. In one weevil the midgut was filled with liquid and it was transparent instead of intransparant. Some of the original structure could be seen and gregarines were inside the midgut. This appears to confirm the assumption that the gregarines affect the midgut and change its structure.

One weevil that did not contain gregarines had a whitish substance in its gut and midgut; it looked a bit like chalk which could be caused by a fungus.

To determine if the gregarines have been present also in formerly collected populations, around ten weevils, which were collected in 2000 and kept in the freezer, were dissected and they looked healthy inside. The midgut could be seen clearly and most of the times it filled up about 1/3 of the whole abdomen and the gut was filled with faeces.

2.1.2 Gregarines: Theoretical background

Gregarinia (commonly referred to as gregarines) are protozoa placed under the phylum Apicomplexa. The gregarines found in our study can be placed in the order Eugregarinida. They are found in the digestive system and body cavities of invertebrates (Tanadal and Kaya 1993). This order is mostly considered to have low virulence to insects (Wülker, 1921, Fuchs, 1915 and Tanadal and Kaya 1993). Mostly the damage in the midgut caused by the gregarines is easily repaired (Tanadal and Kaya 1993). However, in some studies it is concluded that gregarines can have a negative effect on the insect. For both the mealworm, *Tenebrio molitor*, and the black carpet beetle, *Attagenus megatoma*, it has been found that the growth is negatively affected by the gregarines. The American cockroach, *Periplaneta americana,* the cricket *Gryllus campestris*, the American termite, *Coptotermes acinaciformis,* and carabid and scarabaeid beetles can all be killed because of a gregarine infection. On the other hand, some studies suggest that there is a mutualistic interaction between the gregarine and its host and positive effects of the gregarine like increase of growth rate and reduction of mortality have been found (Tanadal and Kaya 1993).

The insects are infected by ingestion of mature oocysts. Sporozoites emerge from the oocyte and penetrate into the midgut epithelial cells or into the

hemocoel and become thropozoites. When the thropozoites have grown, they emerge from the cells (during this process the cells are destroyed) and they develop in the lumen of the digestive tract into gamonts. The gamonts undergo syzygy (association of 2 or more gamonts) and a cyste (gametocyst) is produced around the gamonts. The gamonts produce gametes, which fuse to form zygotes. This is the only diploid stage in the life cycle of gregarines. The zygotes form a thick membrane to form the oocyst and undergo first meiotic divisions, followed by mitotic divisions to produce haploid sporozoites. The oocytes can emerge by bursting the gametocyst wall, or special tubes are produced to spread the oocysts. The number that develops in the host is no more than the number of sporozoites that emerge from the ingested oocytes (Tanadal and Kaya 1993).

The gregarine species which affects *Ips typographus*, are excreted as cyste through the faeces of the beetle (Fuchs, 1915). The sciarid fly, *Trichosia pubescens*, spreads the sporozoites when it lays its eggs or defecates (Tanadal and Kaya 1993).

Gregarines can be found in both larvae (Wülker, 1922) and adults (Purrini & Ormieres, 1981) of *H. abietis*.

Purrini and Ormieres (1981) studied the gregarine *Gregarina hylobii* present in *H. abietis* (collected in Germany) and described the different stages found in the dissected weevils. The weevils survived for six weeks without significant mortality, however later the mortality increased rapidly. Some adults were not as active as the healthy ones and finally stopped feeding. In some cases > 600 gamonts and > 15 gametocysts were found after dissecting these weevils. Triple infections with two other protozoa, *Ophryocystis hylobii* and *Nosema hylobii,* were also observed.

In a study of parasites and pathogens in a Swedish *H. abietis* population, no *G. hylobii* were found (Schabel & Taft, 1988)

Schabel & Taft (1988) studied *G. hylobii* in two species of American pine reproduction weevils (*Hylobius pales* and *Pachylobius picivorus*) and found

gregarines in the posterior portions of the gut and in faeces. A larger proportion of weevils was infected later in the season; 30% of the weevils contained gregarines in mid-March and 75% in September. Almost all the weevils reared in laboratory cages for one month or longer became infected. In the field, the largest number of gregarines found in one weevil was nine, compared to over 200 found in weevils reared in the laboratory.

2.1.3 Gregarines: Impact

A comparison of my observations with the observations and pictures from the study done by Purrini & Ormieres (1981) strongly indicates that the gregarine found in my material was *Gregarina hylobii*. Not much is known about gregarine infections and only two articles have been found about this specific species. Most articles state that a gregarine infection is harmless to the host and do not cause raised mortality, because the host can easily overcome the damage. However Purrini and Ormieres (1981) found less feeding and increased mortality during their study by the infected weevils reared in a laboratory. This is in agreement with my observations.

When the results of this study are interpreted, the infection caused by the gregarines has to be taken into account. The gregarine infection could not be seen from the outside, and moderate infections might have affected weevil behaviour although this was not observed. Thus, the results of the experiments might have been affected even when the weevils appeared healthy.

3. Experiments: Methods and Results

The aim of this research was to find experimental set-ups to test how egg laying and feeding by *H. abietis* are influenced by factors related to previous egg laying by the same species.

I had several research questions (see §1.3), according to which each experimental set-up was designed. The set-up was successively changed when improvements were necessary. In this way there is a lack of replications, since the experiments performed in the beginning of this study were not identical to the experiments performed later. Because of the changes in set up, the methods and results are discussed together. When important adjustments were made between tests, these changes are noted in a box next to the results.

Statistical analyses were performed with the statistical program Minitab (Minitab TM Statistical Software, Release 13.31, Minitab Inc.). A suitable test was chosen for every experiment. The data was checked on homogenous variance and normal distribution. If the data fulfilled the criteria, a parametric test was performed. If the criteria could not be fulfilled, a non-parametric test was done (Fowler et al. 2003; Wiley and Sons). For all analyses, a 95% confidence interval was used.

3.1 Egg laying - no choice experiment

Aim: *To see whether the placing of an egg by a female weevil depends on where another female weevil had placed her eggs.*

3.1.1 General Materials and Methods

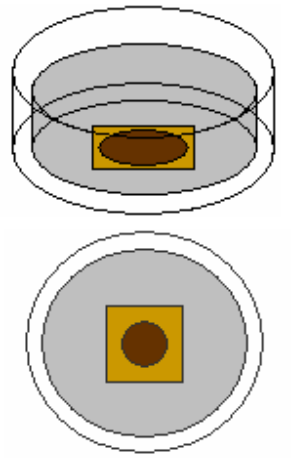

Figure 4: Experimental set-up ○ = Outer petri dish, ○ = Inner petri dish, □ = Bark under petri dish, ● = Bark exposed to weevil.

One hole with a diameter of 2.5 cm was made in the bottom of a plastic Petri dish (in these experiments this is called the inner dish). A piece of bark was cut from a stem piece and was made as thin as possible. The bark was put under the hole, with the outside of the bark to the under side of the Petri dish. A lid of a Petri dish was put under the hole (this is called the outer Petri dish) while another one was used to close the Petri dish, to avoid escaping of the weevils. Around the bark, a piece of wet filter paper was put, to protect the bark from drying out. In this way, the eggs became visible from the underside of the Petri dish, since the bark was too thin to hide egg chambers and eggs (Figure 4).

One female was put in each Petri dish and the next day it was checked whether this first female had laid eggs. If she laid one or more eggs, the female was removed from the Petri dish and the place of the eggs was marked with a red spot on the outside of the underside of the Petri dish. The weevil was kept in the Petri dish for up to two days before this part of the experiment was stopped. Photos were taken from the upper and underside of the bark. Feeding damage was also recorded by drawing a map of the feeding scars by putting a piece of plastic foil over the bark.

A second female was thereafter placed in the Petri dish for 1 or 2 days, depending on the number of eggs laid. When she laid eggs, these were marked with a green spot. Again photos were taken from the upper and underside of the

bark and a map was drawn showing feeding places. In this way the location of feeding and egg laying of the first and second female was determined.

To analyse the data, the pictures of egg distribution and feeding pattern were printed out, and maps from each Petri dish were drawn on transparent sheets. On the maps, the location of the eggs from the first and second female was marked. Another map was drawn to mark the distribution of the feeding scars made by the first female. A third map was drawn to depict the distribution of feeding by the second female. The maps were scanned per Petri dish, and the distances from one egg to another were measured with the digital image-analysing program 'Image' (supplied by S. Karlsson; http://www.sk-biometri.se), as well as the size of the feeding marks.

3.1.2 Results

Hypothesis: The average distance between the eggs laid by the first female (1) and second female (2) is smaller than the average distance between the eggs from the two females (3) (figure 5).

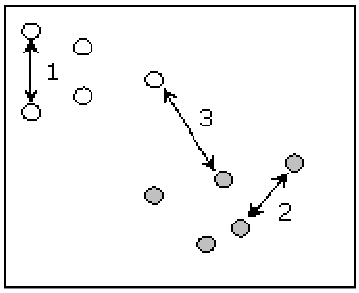

Figure 5: A schematic drawing of a piece of bark with eggs. ○= egg from first female. ●= egg from second female. 1= the distance between eggs from the first female. 2= the distance between the eggs from the second female. 3= the distance between the eggs from the first and second female.

Result: The average distances between the eggs were calculated for the three different groups (Table 1); first female, second female and inter (the distances between the eggs from the first and second female). A one-way ANOVA was accomplished to test if there were differences between the three groups. There were no differences between the groups (degrees of freedom (df) =2; F=0.89; P=0.418), but there was a significant difference between the repetitions (df=21; F=2.89;

P=0.002), indicating variation between the Petri dishes. The hypothesis is not supported; the distance between the eggs laid by one female is not smaller than the distance between eggs laid by 2 different females.

Replication	Egg distances first female (1)	Egg distances second female (2)	Egg distances between females (3)	Number of eggs 1st female	Number of eggs 2nd female
1	6.21	5.35	11.71	5	6
2	12.43	7.61	10.96	5	16
3	3.43	10.37	12.77	3	3
4	-	11.9	6.5	1	2
5	4.55	-	12.4	4	1
6	9.1	11.8	9.6	7	3
7	6.87	8.2	14.75	3	2
8	6.7	-	8.4	2	1
9	7.03	8.77	8.78	4	3
10	16.6	9	11.25	2	2
11	9.3	12.1	10.56	2	2
12	10.3	18.5	9.925	2	2
13	7.9	12.08	10.46	2	8
14	16.4	9.4	22.92	3	2
15	8.1	12.2	10.38	4	2
16	-	11.63	12.53	1	6
17	6.7	1.6	10.225	4	2
18	15.72	19.8	20.57	6	3
19	-	3.23	5.2	1	3
20	19	19.4	15.86	3	3
21	6.85	-	6.48	6	1
22	-	21.6	10.25	1	2
Average	**9.62**	**11.29**	**11.48**	**3.23**	**3.41**
Total				**71**	**75**

Table 1: The average distances (in pixels) between the eggs of the 3 different groups and number of eggs for each female for each repetition.

When a female has to lay her egg on a piece of bark where another female had placed her eggs, there is a possibility that the eggs of the second female are more spread than the eggs of the first female. The second female is restricted in

placing her eggs, and therefore the pattern can be more distributed than the pattern of the first female. Therefore the subjoined hypothesis is formulated.

Hypothesis: The eggs laid by the first female are more aggregated than the eggs laid by the second female.

Method: An aggregation index was calculated by using the nearest-neighbour method (Krebs, 1999). The aggregation index was calculated for each Petri dish for 3 groups; eggs laid by the first female, eggs laid by the second female and the total amount of eggs. Only experiments were used when both females had laid 3 or more eggs. With this criterion, 15 experiments were excluded.

The formulas used were:

$$\mathbf{r_A = \frac{\sum r_i}{n}}$$

r_A = Mean distance to the nearest neighbour

r_i = Distance to the nearest neighbour for individual i

n = Number of individuals in study area

$$\mathbf{\rho = \frac{Number\ in\ study\ area}{Size\ of\ study\ area}}$$

ρ = Density of organisms

$$\mathbf{r_E = \frac{1}{2\sqrt{\rho}}}$$

r_E = Expected distance to nearest neighbour

$$\mathbf{R = \frac{r_A}{r_i}}$$

R= Index of aggregation

Replication	first female	second female	Total
1	0.59	0.45	0.72
2	0.84	0.51	0.47
3	0.45	1.29	1.19
6	1.32	0.86	0.81
9	0.87	0.79	0.62
18	1.64	1.60	1.31
20	2.24	2.80	1.54
Average	**1.14**	**1.19**	**0.95**

Table 2: Aggregation index for the eggs laid by the first female, second female and the total amount of eggs.

Result: A General Linear Model was done, but there were no differences in aggregation for the three groups (df=2; F=0.93; P=0.422), so the hypothesis is not supported, there was no difference in aggregation between eggs laid by the first or second female.

I looked for a relationship between the number of eggs laid by a weevil and the area of bark eaten, the number of feeding marks and the average size of the feeding marks. Possibly the higher the number of eggs correlates to a higher amount of bark eaten, to a higher number of feeding marks, or to a smaller size of the feeding marks. A Pearson test was done to test if there were correlations between the amount of eggs were and the factor which was tested. No significant differences were found (Amount of bark fed: N=22; r=-0.148; P=0.511, number of feeding marks: N=22; r=0.120; P=0.594, size of feeding marks: N=22; r=-0.284; P=0.195), so it cannot be said that there is a correlation between the amount of eggs laid and the area of bark eaten, the number of feeding marks and the average size of the feeding marks.

3.2 Egg laying - choice experiment (first set-up)

Aim: *To investigate if the distribution of eggs laid by a H. abietis female is affected by previous egg laying by simultaneous exposure to bark with and without eggs and feeding scars.*

3.2.1 General materials and methods

Aluminium foil was wrapped around 6 cm long stem pieces of Scots pine cut in half. A square of 1.5 x 1.5 cm was cut out from the foil to expose the bark, in which four small holes had been made. The stem was put in a glass Petri dish and a weevil (Svärdsjö batch) was placed in the Petri dish (10 repetitions).

48 hours after the start of the experiment the females were removed and photos were taken of the stem piece. Feeding damage was also recorded by drawing a map of the feeding places by putting a piece of plastic foil over the stem. A second 1.5 x 1.5 cm piece was cut out of the aluminium foil at 2 cm distance from the first exposed bark area. The area that had been exposed to the first female for two days was called the 'infested' side. The new exposed area with no feeding damage was called the 'clean' side. The infested site was marked with a red dot. 4 deep holes were made in the clean area. One new female was placed in each Petri dish. After 96 hours, these second females were removed and pictures were taken of bark areas. These pictures were compared with the pictures taken after 48 hours, and it was determined how much each weevil had fed and where they had laid their eggs.

3.2.2. Results

For each experiment the number of eggs laid in the 'infested' and 'clean' side was recorded. The column for infested side is divided in 4 columns; eggs laid by the first female, eggs laid by the second female, eggs for which the female which laid the egg is unknown, and the total number of eggs (Table 3).

	Infested side				Clean side
Experiment	First female	Second female	Unknown	Total	Second female
1	4	5	12	21	4
2	0	2	14	16	8
3	11	8	17	36	14
4	16	11	38	65	28
5	3	0	2	5	2
6	6	6	15	27	3
Total	**40**	**32**	**98**	**170**	**59**

Table 3: The number of eggs laid in the 'infested' vs. 'clean' side.

Because of the high number of eggs of unknown origin, no statistical analysis was done for this experiment. However the results in table 6 reveal no evidence for deterrence against the eggs laid by the first female, since the second female also laid considerable numbers of eggs on the infested side.

Because of the difficulty to investigate the origin of the eggs (whether laid by the first or second female) a new set-up was developed, in which it was possible to determine the origin of the eggs. This experiment is described in § 3.3.

3.3 Egg laying - choice experiment (second set-up)

Aim: *To investigate if the distribution of eggs laid by a H. abietis female is affected by previous eggs laying by simultaneous exposure to bark with and without eggs and feeding scars.*

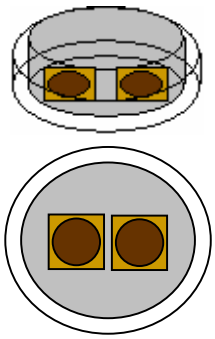

Figure 6: Experimental set-up without upper lid. ○ = Outer Petri dish with wet filter paper, ◯ = Inner Petri dish, ■ = Bark under Petri dish, ● = Bark exposed to weevil.

3.3.1 General materials and methods

The set-up of this experiment is similar with the one described in the egg laying pattern experiment (§ 3.1.1). The differences are that two holes with a diameter of 2.5 cm were made in the bottom of a plastic Petri dish. Two pieces of outer bark were put under the holes (Figure 6). One of the holes was covered with a piece of plastic, which was kept in place with a piece of tape on the underside. When the first female had laid two or more eggs, she was removed and the covered hole was opened. A second female was then introduced and allowed feed and oviposit for two days. All the weevils used in the experiments were taken from the Asa batch.

To measure the feeding damage, the maps (drawn on the plastic foil) were scanned. For each female the amount of feeding was calculated using the program Image.

3.3.2 Results

3.3.2.1 Eggs

The results of the egg laying choice experiment are shown in table 4.

	Infested side		Clean side	
Repetition	First female	Second female	Second female	Total second female
1	14	2	2	4
2	3	0	1	1
3	2	1	1	2
4	4	3	0	3
5	5	2	0	2
6	2	1	5	6
7	4	0	1	1
8	3	0	2	2
9	1	0	1	1
10	2	1	0	1
11	4	1	0	1
12	2	4	0	4
13	6	1	2	3
14	7	2	0	2
15	3	3	1	4
16	4	3	4	7
17	3	2	1	3
18	4	4	3	7
19	3	2	1	3
20	2	2	0	2
21	2	3	0	3
22	3	0	3	3
23	2	3	1	4
24	2	0	1	1
25	5	0	1	1
26	2	1	2	3
Total	**94**	**41**	**33**	**74**
Average	**3.6**	**1.6**	**1.3**	**2.8**

Adjustment
Repetition 1-10:
The same piece of bark of one piece is put under the 2 holes.

Adjustment
Repetition 25 and 26:
The weevils used were taken from the Asa II collection.

Table 4: Number of eggs laid in the choice experiment. Each repetition represents one Petri dish.

Hypothesis: ***The number of eggs laid by the second female in the clean side is higher than the number of eggs laid in the infested side.***

Result: The number of eggs laid on the infested side by the second female was compared with the number of eggs laid on the clean side. A paired t-test showed no significant difference (N=26; T=0.81; P=0.425). The hypothesis is not supported; the second female did not prefer to lay her eggs on a clean piece of bark over a piece of bark in which another female had already laid her eggs.

Hypothesis: The total number of eggs laid by the second female is higher than the number laid by the first female (since the second female has more bark available for egg laying).

Result: The total number of eggs laid by the second female was compared with the number of eggs laid by the first female. A paired t-test was done and no significant difference was found (N=26; T=1.34; P=0.193). Therefore the hypothesis is not supported; there was no difference in the number of eggs laid by the first or second female, having access to different amounts of bark.

3.3.2.2 Feeding damage

The feeding scars for 6 repetitions were scanned, and for each female the amount of feeding was calculated (Table 5). The low number of repetitions is due to a mistake which occurred during the experimental procedure.

	Infested side		Clean side	
Replication	First female	Second female	Second female	Total second female
6	1017.1	105.1	602.7	707.8
7	1107.7	210.1	693.6	903.7
8	1025.4	198.1	691.4	889.5
9	301.6	217.4	658.8	876.2
10	856	258.8	560.2	819
Average	**861.56**	**197.9**	**641.34**	**839.24**
St. Dev.	326	56.7	58.3	80.2
SE mean	146	25	26	36

Table 5: Feeding area (in pixels) recorded in the choice experiment. The results of the choice experiment. Each repetition stands for one Petri dish.

Hypothesis: ***The amount of feeding by the second female is higher on the clean side than on the infested side.***

Result: The amount of bark eaten by the second female from the clean and infested side is compared with a paired T-test. This shows that the second female prefers to feed from the clean side (N=5; T=12.19; P=0.0000) and therefore the hypothesis can be accepted; the second female prefers to eat from a clean side of bark, instead of bark on which another female has already fed and laid eggs. However, the result is based on very few repetitions, and therefore must be seen as an indication.

Hypothesis: ***The second female eats more than the second female (since more bark area is available to the second female).***

Result: The amount of feeding of first and second female was compared with a paired t-test. No significant difference was found (N=5; T=0.15; P=0.889), so the hypothesis is not supported; there was no difference in the amount of bark eaten by the first or second female, having access to different amounts of bark.

3.4 Egg deterrence – choice experiment

Aim: *To determine if chemicals present in the eggs deter H. abietis females from feeding.*

3.4.1 General materials and methods

A piece of Scots pine stem (length 5 cm, Ø 1-1.5 cm) was split longitudinally and aluminium foil was wrapped around each half. Two holes were made in the aluminium foil (Ø 0.6 cm) with 2 cm in between. In one hole, two eggs were rubbed out on the bark, so the contents of the egg was spread over the hole (this side was marked with a red dot, and is named 'treatment'). The other hole was used as a control (marked green). The pieces of stem were put in plastic Petri dishes. One female weevil (Asa batch) was put in each Petri dish. The percentage of the bark area exposed in each hole that had been fed upon by the weevil was recorded after 2, 6 and 24 hours. The number of repetitions differed from four till ten Petri dishes per experiment, dependent on the amount of weevils available.

3.4.2 Results

Hypothesis: Females of H. abietis are deterred from feeding on bark contaminated with contents of conspecific eggs.

Result: The data were analysed in a paired t-test for each time interval. No significant differences were found after 2, 6 and 24 hours (respectively, T=1.29, P= 0.203; T=0.41, P=0.681; T0.52, P=0.604; N=6 for all 3 analyses) in the percentage of bark area consumed; so the hypothesis is not supported, the females were not deterred by bark containing egg elements. The results for each experiment are shown in table 6.

		Number of females fed on T or C						Mean bark area consumed (%)					
		2 hours		6 hours		24 hours		2 hours		6 hours		24 hours	
Experiment	N	T	C	T	C	T	C	T	C	T	C	T	C
1	10	1	1	1	2	4	3	3 %	1 %	3 %	3 %	13 %	6 %
2	4	0	0	1	0	2	4	0 %	0 %	5 %	0 %	12.5 %	22.5 %
3	6	0	1	0	2	2	4	0 %	1.7 %	0 %	5 %	8.3 %	21.7 %
4	10	0	0	1	0	4	8	0 %	0 %	1 %	0 %	14 %	17 %
5	10	2	2	3	2	6	7	1 %	3 %	3 %	4 %	15 %	18 %
6	10	2	3	4	4	8	10	1 %	8 %	13 %	8 %	42 %	42 %
Average/exp.		0.8	1.2	1.6	1.6	4.3	6	0.8%	2.3%	4.2%	3.3%	17.5%	21.2%

Table 6: Feeding by *H. abietis* females on bark treated and not treated with two splashed *H. abietis* eggs Feeding recorded after 2, 6 and 24 hours. N= number of repetitions. T= Treatment side and C= Control side.

Adjustment
- Experiment 1: Females used from Svärdsjö batch.
- Experiment 1 & 2: 3 eggs were rubbed out on the treatment side.

3.5 Faeces deterrence - choice experiment

Aim: *To determine if chemicals present in the faeces deter H. abietis females from feeding.*

3.5.1 General materials and methods

To test the effect of presence of faeces on feeding, the method described in §3.4.1 was used. In experiment 7, 8 and 9 faeces were removed from the eggs ('egg faeces') and suspended in tap water (experiments 7 and 8) or methanol (experiment 9). The treatment is the side on which the egg faeces solution is applied and as a control tap water or methanol was used, dependent on the liquid in which the faeces was dissolved.

In experiment 10 and 11 faeces was used that had not been deposited in connection with oviposition ('normal faeces'). The faeces was collected from 10 females during 24 hours and was dissolved in methanol. The results of the experiments are shown in table 7 and 8.

		2 hours		6 hours		24 hours	
Experiment	N	T	C	T	C	T	C
7	10	0	1	1	2	5	8
8	5	4	2	4	4	4	5
9	5	0	1	1	3	3	5
10	8	0	2	0	6	7	7
11	27	2	3	3	3	18	22

Table 7: Number of females that had fed on bark treated (T) and not treated (C) with extracts of *H. abietis* faeces. Feeding recorded after 2,6 and 24 hours.

		2 hours		6 hours		24 hours					
Experiment	N	T	C	T	C	T	C	P-value		T-value	
7	10	0 %	2 %	0.5 %	6 %	16.5 %	38 %	0.08	0.06	2	2.01
8	5	13 %	10 %	13 %	26 %	32 %	42 %	0.58		0.61	
9	5	0 %	2 %	4 %	14 %	14 %	52 %	**0.02**		3.92	
10	8	0 %	3.75 %	0 %	13.8 %	20 %	41.3 %	0.17	0.21	1.53	1.27
11	27	1.5 %	0.7 %	1.9 %	3 %	19.3 %	22.2 %	0.62		0.5	

Table 8: Mean bark area consumed from bark treated (T) and not treated (C) with extracts of *H. abietis* faeces. Feeding recorded after 2, 6 and 24 hours. The P and T values are shown for the observations after 24 hours.

3.5.2 Results

Hypothesis: ***Female H. abietis are deterred from feeding from bark containing faeces (deposited in association with the egg).***

Result: A paired t-test was done to test the preference of the females. Experiment 7 and 8 had the same set-up and the data after 24 hours were tested with a paired t-test. No significance difference was found, however, there is a tendency that the weevils prefer the control side over the side with 'egg faeces'.

For experiment 9, a significant difference was found. The weevils prefer the control over the faeces treatment.

For experiment 10 and 11, also a paired t-test was done for the results after 24 hours, but no significant differences were found.

The results are not consistent, but there is a tendency that the weevils prefer the control over the faeces treatment, as the hypothesis predicts. A former study (Nordlander et al. 2000, Borg-Karlson et al. in manuscript) shows a deterrent effect for methanol extracts of faeces, however the effect was stronger in previous tests than in the results shown in this study.

3.6 Clean vs. contaminated egg choice experiment

Aim: *To compare the response of H. abietis when exposed to 'clean' eggs which had been laid in an empty Petri dish vs. eggs contaminated with faeces, laid in bark.*

3.6.1 General materials and methods

In order to obtain clean and contaminated eggs for the test, 20 female weevils were taken from the Svärdsjö batch. Ten of these were put in an empty Petri dish (clean eggs). The other 10 were put in a glass Petri dish with 2 pieces of stem (cut in half) with aluminium foil wrapped around the ends (contaminated eggs). The testing method varied with time, because there were problems with the egg laying of the weevils.

3.6.2 Methods and Results

Experiment 1

Method

15 eggs (collected from buckets in which they are stored) were put in a Petri dish together with two female weevils, which had been starved for 2 days.

Results

The next day, two eggs looked dehydrated. It is unknown if the eggs were damaged by the weevils or if they dried out naturally.

Experiment 2

Method

One egg was collected from the bark ('contaminated' egg) and one egg from the empty Petri dish ('clean' egg). The eggs were put on different sides of a Petri dish, supplied with a low barrier (± 0.5 cm) in the middle preventing the eggs from rolling over to the other side. A weevil (starved for 4 hours) was put in the Petri dish with the eggs and stayed there for 20 hours. This experiment was repeated four times.

Results

The next day none of the eggs were damaged. One weevil laid a small egg with the faeces attached on it.

Experiment 3

Method

The same set-up as experiment 2, but the weevils were starved for three days and stayed in the Petri dish for 24 hours. The experiment was done twice.

Results

The next day both clean eggs looked damaged, they shrank and seemed dehydrated, whereas the contaminated eggs remained undamaged.

Experiment 4

Method

Three clean eggs were collected from the paper which is closing the water tube from the storage bucket. The contaminated eggs were taken from pine bark in the Asa storage bucket. The females (Asa batch) were starved for 30 hours. They were put in a Petri dish, which was divided by a piece of tape, and they remained there for 24 hours. The experiment was repeated 3 times.

Results

In the 3 Petri dishes the clean eggs shrank. Of the contaminated eggs, one was intact after 24 hours, one was eaten by the weevil, and one looked dehydrated.

In the experiments it was difficult to see whether the eggs were damaged by the weevil, or dehydrated. In experiment 3, the clean eggs looked damaged, while the contaminated eggs stayed intact. It is difficult to draw conclusions from these experiments; a more suitable set-up must be designed to test this.

3.7 Observations of the egg laying behaviour of Hylobius abietis

3.7.1 First phase: Making the egg chamber

The egg laying process always starts with making a feeding mark. The weevil starts to make a deep hole in the bark, which goes down to the xylem and continues sideways into the phloem (Figure 7). When making the egg chamber, the female puts her body in a typical position. She leans forward and puts her snout under her body into the bark (Figure 8). When the excavation of the egg chamber is completed, she turns around and starts phase 2. It is difficult to estimate the duration of phase 1, since its initiation is hard to establish.

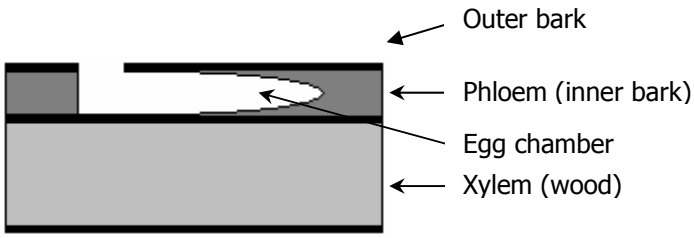

Figure 7: A schematical drawing of a section of pine tree with egg chamber after phase 1.

Figure 8: Female weevil excavating an egg chamber. Photo: M. Munneke

3.7.2 Second phase: Laying the egg

When the weevil has turned around, she extends 2 sense organs (Figure 9), which are situated at the end of the ovipositor (egg-laying tube). With these sense organs she explores the bark and tries to locate the egg chamber. When the egg chamber is found, the female extends her ovipositor in the egg chamber. The ovipositor is a flexible transparent organ, which can be extended from the backside of the abdomen. The egg is laid in the egg chamber, most of the times together with some faeces. After laying the egg she turns around again and starts phase 3. The egg-laying phase takes around one minute.

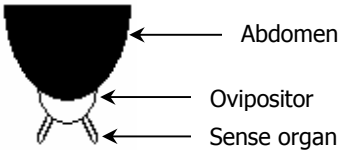

Figure 9: A schematical drawing of the abdomen and ovipositor with sense organs of *Hylobius abietis*.

3.7.3 Third phase: Closing the egg chamber

After turning, the female first pushes her snout into the egg chamber, probably to push the egg (and, if present, the faeces) deeper into the egg chamber. Then she starts nibbling around the opening, where she collects pieces of bark from the sides and transfers them to the opening. She keeps doing this till the opening is closed. It is not known whether she uses saliva or other liquids to keep the pieces of bark together. Sometimes pieces of faeces are present in the layer of bark pieces, which the weevil makes to close the egg chamber, but in other instances there are no faeces present. This layer that closes the egg chamber is often referred to as frass plug. When the frass plug is ready, the female stops and moves away. This third phase takes around 10 minutes to complete. A picture of a completed egg chamber can be seen in Figure 10.

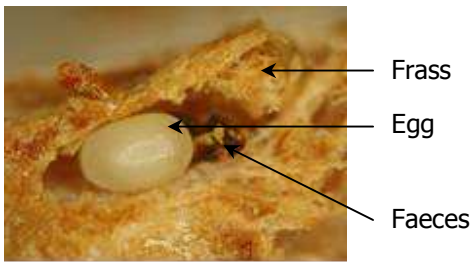

Figure 10: Completed egg chamber viewed from above. Photo: M. Munneke

4. Discussion

Hylobius abietis is a major pest of planted conifer seedlings, and therefore it is important to find a suitable control method. Since chemical control methods are not always a good solution (regarding to negative effects on human health and the environment), there is an interest in new biological control methods. In this study, I looked at the deterrent effects of pine weevil faeces, which are deposited together with the egg. It was expected that the faeces would have a deterrent effect, and that female weevils would avoid feeding and laying eggs near eggs of other females. Because this was a pilot study, the emphasis was on designing good experimental set-ups. Therefore the numbers of repetitions were not high enough for making firm conclusions in many instances. Moreover, the experiments suffered from problems with protozoans infecting the weevils used in the tests.

4.1 Discussion: Experimental set-up

To investigate if the presence of an egg influences the position of the egg laid by a second female, choice and no-choice experiments were done. The set-up of the no-choice experiment (§ 3.1) seems suitable. The placement of the eggs can be seen on the underside of the Petri dish, and the origin of the eggs can be determined. However, it can be questioned if the area of exposed bark is not too small (surface = 4.9 cm^2); the second female does not have much possibility to lay her eggs far from previously laid eggs. On the other hand, if the second female would be deterred from the eggs present, she would not lay her eggs at all, lay them as far away from the present egg as possible or lay them on the plastic of the Petri dish (this was seen in the experiment described in § 3.6, where the females had no bark or other medium to lay their eggs on, and the eggs were laid on the Petri dish or filter paper). Another important point is that

the time the two females spend in the Petri dish must be the same, to make comparison of the results possible.

To investigate whether the female weevil prefers a clean piece of bark over a piece of bark in which another female had laid her eggs, choice experiments were performed.

The choice experiment described in § 3.2 turned out to be insufficiently informative. It was difficult to see which egg was laid by which female, and therefore it can not be determined if the egg laying behaviour of second female is influenced by the presence of eggs. With the improved set-up described in § 3.3 it was possible to distinguish the origin of the egg. In this experiment the weevils also have the choice to lay their eggs on a clean piece of bark if they are completely deterred from egg-laying in the infested bark. For this experiment it is also important that the 2 females spend the same time interval in the Petri dish. This set-up appears as a suitable experimental set-up to test the preference between clean bark and bark previously exposed to another ovipositing weevil.

To study the feeding deterrent effect of the egg and its faeces a choice test was done. The set-up was not adjusted during the test series, only the treatments were changed. I first tested deterrence against the egg content alone. Later the deterrence against the 'egg faeces' and 'normal faeces' was tested.

To investigate if there was deterrence against only the egg with the faeces attached to it (without bark), the set-up of the 'Clean vs. contaminated egg' (§ 3.6) experiment was designed. It turned out that the results of this experiment were not conclusive, since it was difficult to see if the damaged eggs were just dehydrated or if they were damaged by the weevil. Although it is known that female weevils sometimes damage eggs, it is not known to what extent they prefer to use eggs as food. To improve the method of this experiment, a higher

humidity must be maintained during the experiment, to prevent drying out of the eggs.

Suitable experiments to look if the egg laying behaviour of the female weevils is influenced by the presence of eggs from another weevil, are the choice and no-choice tests described in § 3.1 and §3.4. In these experiments the weevils are allowed to make an egg chamber and it can be seen where the eggs are laid. An improvement of this experiment could be to increase the area of exposed bark to allow the weevils to respond within a larger range of distances from a previously laid egg.

If deterrence is found against the egg together with faeces, each component should be tested separately. This can be done in the choice test described in § 3.4 and §3.5.

4.2 Discussion: Results

Is the feeding behaviour of the weevils influenced by the presence of feeding marks from another weevil?

I looked at the feeding behaviour, because the egg laying process in *H. abietis* always starts with a feeding mark. However, it is not possible to distinguish a common feeding mark from a mark which is made as the first step of excavating an egg chamber.

The no-choice egg laying experiment (§3.1) shows no influence of previous weevil activity on the amount of bark eaten, the number of feeding marks or on the size of the feeding marks.

In the choice egg laying experiment (§3.3), there was found that a female prefers to eat from a clean piece of bark instead of bark on which another female has already fed and laid eggs.

According to these two experiments, it appears like the female weevils prefer an uninfested piece of bark. However, in a no-choice situation, they are not deterred by the feeding marks of other females, and it occurred often that a second female started her feeding adjacent to where the first female had already eaten. These results indicate that no deterrent compounds are released when a feeding mark is made.

Is there deterrence against eggs (with or without faeces attached)?

The results of the egg laying experiments (§3.1), do not show that the egg laying of a female weevil is influenced by the presence of eggs of another female; the distances between eggs of the same female is not different than the distance between the eggs laid by different females, there is no difference in the number of eggs laid, and there is no difference in aggregation of the eggs. This indicates that there is no deterrence against eggs laid by another female.

The choice tests described in §3.2 and §3.3 show no preference for the side in which a clean piece of bark is exposed compared to bark in which another female had already laid her eggs.

No deterrence of egg contents spread on bark was shown in the choice test with clean bark. However, similar choice tests with faeces gave deterrent effects. In tests with 'egg faeces' dissolved in water there was a tendency of preference for the control side ($P=0.06$). When egg faeces were dissolved in methanol, a significant preference for the control side was found. A strong feeding deterrent effect of pine weevil faeces has previously been found (Nordlander et al. 2000, Borg-Karlson et al. in manuscript).

According to the experiments, no clear deterrent effect of previously laid eggs is found. The egg laying behaviour appears not to be influenced by the presence of eggs from other females. This is remarkable, because it was expected that the

presence of eggs from other females would have an effect. It is known from other insect species that deterrent substances are attached to the egg to reduce competition (Blum & Hilker, 2002, Kozlowski et al. 1983, Ferguson & Williams, 1991). Since the *H. abietis* female adds faeces to her eggs, it has been suggested that they serve to protect the eggs from predation by conspecifics or to reduce larval competition (Borg-Karlson et al. in manuscript). Overall, the pilot experiments reported here do not indicate that adding faeces deter other female weevils or that other deterrent compounds are added. However, in the choice experiments with faeces alone, a feeding deterrent effect was observed.

A general conclusion, based on my results, is that no avoidance of eggs or feeding marks from other female weevils exists. However, some of the results indicate a feeding deterrent effect of faeces, when applied to pine bark. Because the presence of faeces besides the egg had no clear effect on feeding or oviposition by other *H. abietis* females, also other explanations for depositing faeces in the egg chamber should be investigated. A possibility could be that the faeces provide bacteria which contribute to a better condition of the larvae.

One factor that has to be properly considered in future work with *H. abietis* is the effect of gregarines. During the experiments many infected weevils were found in the breeding buckets. Probably the gregarines influenced the behaviour of the weevils. For example, faeces deterrence test (§ 3.5) has been done before (Borg-Karlsson et al. in manuscript), and a clear deterrence against the faeces was found. A few weevils which were used in this experiment were dissected after the experiments, and gregarines were present in all the weevils.

Observation of egg laying behaviour is an interesting topic in research on *Hylobius abietis*, since not much is known about it and there is a possibility that a control method can be developed which is based on results from studying the

egg laying behaviour. Therefore it is important to further investigate the egg laying behaviour and to follow up on the experiments described in this study.

5. Literature

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