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The effects of herbivory by *Pieris brassicae,* on pollinator behaviour and nectar production in *Brassica nigra.*

No: 08.23
Name: Cindy ten Broeke
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Thesis/Internship ENT 80436
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Abstract

There are many interactions between plants and insects. Two important interactions are herbivory and pollination, which can affect the selective pressure in the evolution of plant responses and traits. Herbivores affect plant fitness by reducing its resources and damaging reproductive tissue. Plants therefore have evolved multiple strategies to defend themselves against these herbivores. Pollinators also influence plant fitness by transferring pollen from one plant to another, affecting its reproduction. Plants have evolved several strategies to attract effective pollinators, thereby optimising their pollen transfer.

Both herbivory and pollination by insects have been investigated extensively. However, the effect they have on each other is far less studied. Herbivory can affect pollinator cues indirectly, by decreasing flowers size and/or number, decreasing nectar and/or pollen production, and directly by damaging the flower by florivory. Several studies showed that plants with herbivores overall receive less and shorter visits of pollinators, compared to plants without herbivores.

In this research the effects of herbivory by *Pieris brassicae,* on the nectar production and pollinator behaviour in *Brassica nigra,* was investigated. The study system consisted of one plant species, *B. nigra*, one herbivore species, *Pieris brassicae* (caterpillar), and three pollinator species of *B. nigra, P. rapae*, *Apis mellifera* and *Episyrphus balteatus*.

It was shown that feeding by *P. brassicae* on the *B. nigra* plants, affected the nectar production; the amount of nectar was lowered due to herbivory. During nectar analyses, glucosinolates were found in the nectar of *B. nigra*. Herbivory by *P. brassicae* also affected the behaviour of the three pollinator species. The number of visits by *E. Balteatus* and *P. rapae* was lowered due to herbivory. However, the number of visits by *A. mellifera* did not change due to herbivory. The duration of visits was shorter for all the three pollinators due to herbivory.

This study showed that there is an important interaction between herbivory and flower visitation by pollinators, which earns more research.

Preface

Since I was a little child, I have been fascinated by nature and its inhabitants. As a child I often went to ponds with a fishnet, together with my grandfather, to catch all kinds of insects. These insects were put in an aquarium, and we could spent hours watching their behaviour.
In 2003 I started my study Biology at the University of Nijmegen. After a few weeks I kept my first pets in my student room, a rabbit and walking sticks. Nowadays I keep a lot of animals, including some insect species.

Doing a water macro-fauna Mcs. thesis at the University of Nijmegen, increased my interest for insects, and I knew my next Mcs. thesis had to be one with insect too. By following the course Fundamental and Applied Aspects of the Biology of Insects, at the University of Wageningen, I came in contact with the Ento family and the interesting research they were doing. After the course I decided to do my Msc. thesis at the department of Entomology. For this Msc. thesis I researched the effects of herbivory by *Pieris brassicae* on the nectar production and pollinator attraction of *Brassica nigra.*

The time I spent in Wageningen at the department of ecology was a great time and I have learned a lot. Therefore I want to thank my supervisors, Maaike Bruinsma, Joop van Loop and Marcel Dicke, for giving me the opportunity to do entomological research and helping me out during my thesis. Without the rearers, my research would be impossible. Therefore I want to thank Leo Koopman, Frans van Aggelen, André Giddink and Bert Essenstam, for providing my study organisms. I also want to thank Willem Boot and Johan Calis of *Inbuzz,* for providing me a bee colony. Furthermore I want to thank every one of the department of Entomology for all their help and the great time I had during my thesis.

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1. Introduction

1.1 Plant-insect interactions

Insects are the most dominant taxonomic group, containing about 50 % of all described species. They can be found almost everywhere around the world and have adopted a great variation of adaptations to the variable environmental conditions. They affect many other species and play important roles in ecosystems, by being an important food source for many other organisms, for example (Schowalter, 2006).

Plants are the second dominant taxonomic group after insects and are the biggest contributors to the biomass on land. Of all insect species described, almost half feed on living plant tissue (Schoonhoven *et al*., 2005). There are various relationships between plants and insects, ranging from mutualism to parasitism. For most insects, plants are not only for food, but also to live on. Plants provide shelter, food and oviposition sites for insects (Panda & Khush, 1995). Insects can feed on various structures of plants, for example leaves, fruit, pollen, nectar, plant sap etc.

1.2 Plants and insect herbivory

Plants can provide a food source for insects. Only nine insect orders of the 29 living orders, exploit living tissues of higher plants for food. The orders are Coleoptera, Diptera, Lepidoptera, Hymenoptera, Hemiptera, Orthoptera, Collembola, Plasmida and Thysanoptera (trips).

About 18 % of the terrestrial and 51% of the aquatic plant biomass is consumed by herbivores. Therefore this is an important biotic interaction, but also a potential problem for agriculture (McCall & Irwin, 2006). The damage done to food by insects costs farmers and consumers millions a year. In the USA, 13% of the crops are lost to insects before harvesting (Schoonhoven *et al*., 2005).

To successfully exploit plants, insects had to overcome some problems in the evolution, like problems of desiccation, attachment and food (capturing and digestion) (Strong *et al*., 1984). Their diet for example, is based on the structure of their mouthparts. There are several functional groups of herbivores distinguished based on their mouth structure, making it possible to exploit different structures of plants. For example, some species have chewing mandibles to chew plant tissue, while others have piercing mouthparts to suck plant fluids (Panda & Khush, 1995). The chewers consume leaves, stems, flowers, pollen seed and roots. Insects which feed on leaves of plants are often referred to as foliar herbivores. This kind of herbivore will be used in this research. They reduce the photosynthetic area of the plant, thereby decreasing its resources (Mothershead & Marquis,

2000), consuming 50-150 % of their dry body mass a day (Schowalter, 2006). Herbivory on flower buds and flowers is often revered to as florivory. It affects both female and male plant fitness, by directly consuming the gametes, pistels or/and stamen, and thereby consuming resources and reducing flowers (McCall & Irwin, 2006). Furthermore, root herbivores damage roots, resulting in a declining ability of uptake of water, nutrients and minerals, thereby affecting the above part of the plants (Schoonhoven *et al*., 2005). Seed predators and frugivores consume reproductive tissue of plants (Schowalter, 2006). Miners feed between plant surfaces. Gall-forming insects feed within the plant, inducing abnormal growth of plant tissue, and providing nutrients and shelter for the insect causing the abnormal growth induction (Schoonhoven *et al*., 2005). Piercing/sucking is one of the most primitive ways to feed on plants. These sap-suckers feed on plant fluids (Jolivet, 1998).

Herbivores can either be generalists, or specialists. Monophagus insects only feed on closely related plant species, while oligophagus insects feed on plant species belonging to the same family. Polyphagus insects are generalists which feed on plant species from distinguished families (Schoonhoven, 2005).

Herbivory has a negative effect on almost all plant species. Depending on the plant species, they respond differently to herbivory. For example, some species can lose 10% of their leaf area, while others can lose up to 25% of their leaf area without a decrease in reproductive success, expressed in seed set (Strauss & Agrawal,1999). The figure below shows the direct and indirect effects of herbivores on plants (figure 1.1).

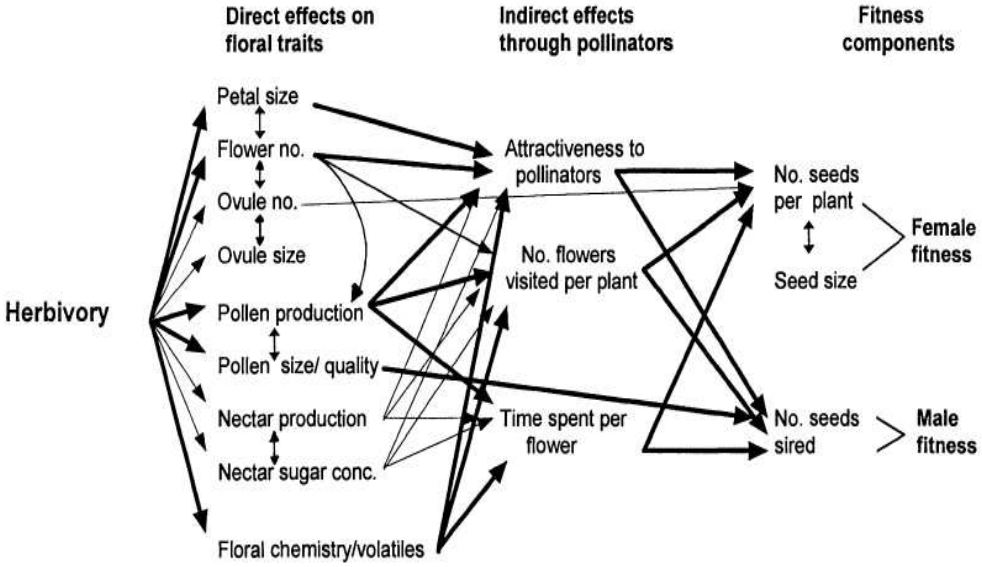

Figure 1.1: A summary of the direct and indirect effects of herbivory on plants (Strauss, 1997).

Direct effects

The main effect of herbivory is reduction of plant biomass, and thereby decreasing the photosynthetic area of the plant. This causes a decrease in resources left for the plant, thereby changing the allocation of these resources to different plant parts, and reducing plant growth (Mothershead & Marquis, 2000).

The stage of the plant, in which herbivory takes place, is important to determine the effects of herbivory on the different plant structures. When herbivory occurs at an early stage, for example at the development of the reproduction structures, differential effects on female and male reproductive tissue may occur. Damage to the young leaves can delay flowering, reduce petal size, lower pollen and nectar production and may result in smaller flowers (Strauss *et al*., 1996; Mutikainen & Delph, 1996), while herbivory after the inflorescence production influences seed size and/or production. If pollen do not receive enough resources at production, fertilisation with these pollen will be affected in terms of pollen-tube growth rate. This pollen-tube growth rate will be slowed and seed siring with these pollen will not occur, when other 'healthier" pollen are available (Mutikainen & Delph,1996; Quesada *et al*., 1995).

Indirect effects

Herbivory affects the pollinator cues, by decreasing flowers size and/or number, decreasing nectar and/or pollen production and directly damaging the flower by florivory (Strauss & Murch, 2004). These changes in flower cues can cause a change in the pollinator composition visiting a plant, because different insects use different cues (Lethilä & Strauss,1997). Pollinator cues are described in more detail in the *pollinator* section of the *introduction*. Plants with herbivores overall receive less visits and shorter visits of pollinators, compared to plants without herbivores (McCall & Irwin, 2006). This reduction in pollinator visitation and effectiveness can cause a reduction in fruit set, due to reduced pollen transfer and thereby reduced seed seering. However, this only occurs in plants that are pollinator limited (Mothershead & Marquis, 2000; Strauss, 1997). The experiments of Lethilä and Strauss (1997) also showed that the pollinator visitation was not limiting seed production and thereby was not limited by pollen. The explanation they proposed was that probably the quality of seeds differed, although they did not examine this.

1.3 Plant defence

To prevent the negative effects of herbivory, plants have developed a range of strategies to defend themselves against their attackers, they can either tolerate the herbivores or escape their attacks. However, the selective ability of insects, such as detoxification sequestration and avoidance, enables them to overcome these plant defence mechanisms and allows

limited feeding (Panda & Khush, 1995). Sometimes the defence against one herbivore can lead to the attack of another species, because some specialist herbivores are attracted by high secondary compound concentrations (Strauss *et al*., 1999).

There are three main defence strategies; tolerance, direct defence and indirect defence (Kessler & Baldwin, 2002). These defences can either be constitutive or inducible, where constitutive is always expressed in the plant, and induced only when needed, after an herbivore attack (Arimura *et al*., 2005). Induced defences are often cost saving, because, for example, plants do not have to produce secondary compounds all the time, just when needed (Baldwin, 1998). However, plants without constitutive defences, are not protected all the time, and inducing responses takes time.

Tolerance

The tolerance of a plant to herbivory is influenced by biotic and abiotic factors. Plants gain enhanced tolerance by increasing the net photosynthetic rate after damage, creating large carbon reservoirs in roots and in case of damage they are able to transport this carbon to the shoots. On the contrary, plants that are pollinator limited, experience reduced tolerance compared with plants without pollination limitation (Strauss & Agrawal,1999). Furthermore, when enough resources, like nutrients, are available, plants can compensate for the damage caused by the herbivores (Strauss & Murch, 2004; Meyer, 2000).

Direct defence

Direct defence, or direct induced responses, are the rapid change in the plants biochemistry, physiology or morphology, and leads to a direct reduced quality of the host for herbivores, thereby increasing the plant fitness in environments with herbivores (Kessler &Baldwin, 2002; Stout, 2007). Induced plant responses are comparable with an immune system, they reduce the performance of the herbivore (Agrawal, 1998). Direct defence, however, can also be constitutive (Arimura *et al*., 2005).

Trichomes (plant hairs) are a form of morphological defence. Non-glandular trichomes limit the access to the surface of the plant and thereby limiting herbivores to feed on it, but also oviposition is deterred. Glandular trichomes, on the other hand, secrete a sticky matter, causing insects to stick in it and die (Karban & Baldwin, 2007), and/or secrete allelochemicals to deter herbivore feeding (Schoonhoven *et al*., 2005). Furthermore, surface waxes can contain allelochemicals, which are deterrent for insects (Stout, 2007), and reduce the grip of insect herbivores to the plant surface (Schoonhoven *et al*., 2005). Finally, a thickened tissue can limit the entrance for insects to feed.

Chemical defences can include repellent volatiles, that for example prevent oviposition by herbivores, as well as growth and feeding inhibitors (Stout, 2007). Protein

inhibitors inhibit the insects digestive enzymes. Toxic primary or secondary compounds (e.g., alkaloids, terpenoids, phenolics) can poison generalist herbivores, thereby forcing specialists to invest resources in detoxification mechanisms that in turn incur growth and development costs. (Kessler & Baldwin, 2002). Plants can also show a hypersensitive response when attacked by pathogens, but some species also do this in case of herbivory, by creating a necrotic zone under the egg of the herbivore within 24 hours after oviposition. Water is drawn from the egg and it will fall of the leaf (Shapiro & De Vay, 1987). Some plant species have secondary compounds, like alkaloids in their floral structures. For example *Castilleja indivisa* (Indian paintbrush) has alkaloids in its calices and floral bracts. The adults of herbivores oviposit on these structures, and when the larva hatch they start eating these structures and die because of the high alkaloid content (Adler, 2000).

Indirect defence

Indirect defence contains plant traits that attract the enemies of herbivores, predators and parasitoids, that can be inducible or constitutive (Arimura *et al*., 2005). Volatiles may be produced to attract the herbivore enemies, and production can be induced by several herbivores, like chewing- and sucking folivorous herbivores, leaf miners, root feeders and oviposition. These volatiles can either be emitted at the damaged site of the plant, or other plant tissue. Different volatiles attract different enemies for different herbivores, where herbivores are recognised by plants through elicitors from oral and oviduct secretions of herbivores. Extra floral nectar is also produced for carnivores, like ants, to feed on, and in exchange they protect the plant against attackers (Heil, 2004).

Glucosinolates

Glucosinolates, or mustard oils, are secondary compounds that appear in all species of *Cruciferae* and some other plant species (van Etten & Tookey, 1979), and cause the strong flavours in broccoli, cabbage and other *Brassicasae* vegetables. Glucosinolates are thought to have multiple functions. They are a defence mechanism against generalist herbivores, by exhibiting outright toxicity, inhibiting growth and feeding deterrence, and a agent for host choice by specialist herbivores (Moyes *et al*., 2000; Halkier & Gershenzon, 2006).

Glucosinolates appear in different structures of plants, like seeds, roots and leaves (van Etten & Tookey, 1979). However, it is not known whether they also appear in nectar. There is some evidence that glucosinolates travel through the plant by long-distance transport. Thereby, glucosinolates posses physicochemical properties required to travel in the phloem, and they are found in the phloem sap and using radiolabbeling it has been shown that they travel from the leaves to the seeds (Halkier & Gershenzon, 2006; Brudenell *et al*., 1999).

1.4 Signalling pathways

The inducible direct and indirect defences are activated by different signal transduction pathways in the plant, involving the octadecanoid pathway with jasmonic acid, the shikimic acid pathway with salicylic acid and the ethylene pathway, which are the main signalling pathways (Schoonhoven *et al*., 2005). Not only the mechanical wound which herbivores cause, is responsible for the induction of the signalling pathway for induced defence. Several studies showed that mechanical damage only, can not mimic the responses of a plant after real herbivory (Arimura *et al*., 2005; Poecke & Dicke, 2002; McCloud & Baldwin, 1997). Although, there are plant species known that only need mechanical damage. For the Lima bean (*Phaseolus lunatus)* the damaged done by a mechanical caterpillar (MecWorm), was sufficient enough to induce the defence response in the plant (Mithöfer *et al*., 2005). On the contrary, elicitors from oral and oviduct secretions play an important role in the induction of the plant defence pathway (Dicke *et al*., 2002), probably in inducing the volatile emission to attract predators(McCloud & Baldwin, 1997).

1.5 Nectar

Nectar is a sugar-rich substance, produced by many plant species, that manipulate their biotic pollinators into transporting pollen (Rhoades & Bergdahl., 1981). Although, the evolution of nectar is not really known. There are theories that first pollen were the attractants and rewards for pollinators, and later plants began to produce a cheaper reward, named nectar. However, some scientist believe that first floral secretions were the rewards and attractants for pollinators, that later specialised into nectar, and therefore nectaries could be originated as excretory organs to get rid of flower superfluous liquid (Pacini & Nicolsen, 2007).

Nectaries are specialized tissues that secrete nectar. Two forms of nectaries can be distinguished. The floral nectaries, located in flowers, that function as attractants for pollinators. The extra floral nectaries, located at stems, that function as attractants for predators of herbivores, which often involves ants (Pacini & Nicolsen, 2007).

The origin of nectar lies in the phloem sap of the plant. However, the components of which nectar consists varies widely. Water is always abundant in nectar and determines the sugar content, but is also an important water supply for a pollinator in dry conditions. Furthermore, carbohydrates are the most abundant components in nectar, and the most important are sucrose, glucose and fructose. The concentration of these sugar in nectar ranges from 7-70%. Besides, this component forms the primary energy source for pollinators, and derives from photosynthesis in the nectaries or other parts of the plant. Amino acids and proteins are the most abundant after sugars, and may play a role in taste preferences of

insects and their nutrition. They determine the dependency of a pollinator on other food sources. Furthermore, antioxidants are involved with the nectar homeostasis. Sometimes lipids are abundant in nectar and may act as a high energy resource for the pollinators. Finally, terpenoids are involved with floral scents (Pacini & Nicolsen, 2007).

Nectar can also contain (toxic) secondary compounds, which are mainly for herbivore resistance, but may also be for selecting the pollinators which visits a plant (Rhoades & Bergdahl, 1981; Adler, 2000). Unwanted pollinators like nectar thieves, robbers and inefficient pollinators can be deterred by the secondary compounds in nectar (Liu *et al*., 2007). For the pollen of plants should be delivered to flowers of the same species, otherwise they are wasted. Therefore, by selecting their pollinators, plants gain enhanced efficiency of pollen transfer. For rare plants this mechanism would increase the successfulness of pollen transfer, by producing highly defended large rewards (Rhoades & Bergdahl, 1981). The cues that pollinators use to choose plants with secondary compounds however, are unknown, but some scientists suggest that the floral display or the amount and accessibility of the nectar are some of the cues involved (Adler, 2000). Although others believe that secondary compounds in nectar may not even have a function, but is a consequence of the production of secondary compounds in other tissues (Singaravelan *et al*., 2005).

The production of nectar brings costs for the plant and it will not produce more than necessary. Experiments, for example, have shown that plants produce more nectar, when it is removed, than plants where nectar is not removed. However, the plants which produced more nectar, also showed a decrease in seed production. So there is a trade-off, between extra fertilized seed by enhanced nectar production, and reduction in seed production because of enhanced nectar production (Pyke, 2000).

1.7 Pollinators

Plants can reproduce in various ways, i.e. vegetative by cloning, or sexual by pollen transfer. Pollination contributes to genetic recombination and survival of plant species in heterogeneous environments. Pollen transfer is accomplished by several mechanisms, which become more important for reproduction with the increasing separation of male and female structures and the increasing of plant individual isolation (Schowalter, 2006), and can either be biotic or abiotic. Most angiosperms (flowering plants) rely totally or partly on biotic pollinators, and not on abiotic pollinators like wind and water. Biotic pollinators include birds, bats and insects. Pollinators contribute to the production of fruit and seeds that support associated food webs. Herewith, the visitation of pollinators affects the amount of seed set (Brody, 1997). Many pollinators are generalists, they eat whatever is available, while the specialist pollinators are adapted to exploit a particular plant species or floral characteristic,

that exclude other (generalists) pollinators. Although, all pollinators feed on pollen and/or nectar (Schowalter, 2006).

Pollinators use visual and olfactory cues to locate, recognize and discriminate flowers. Examples of visual cues are flower colour, number, size and shape. Colour is an important cue and is easily memorised by associating it with reward. However, the floral pigments and defence compounds share precursors (Strauss, 1997). Thereby these pigments can act as defence molecules for stamen and ovaries, and also act as attractors of petals (Herrera *et al*., 2002). Flower shape is another visual cue, where pollinators distinguish radial and bilateral symmetry, and the perfection of the symmetric shape (Schoonhoven *et al*., 2005). Furthermore, some pollinators prefer plants with big and many fruits, which could mean other pollinators favoured this plant before and more nutrients are available for the plant (Herrera, 2000). Finally, flower odour is also an import cue for pollinators. The combination of visual and olfactory cues are better remembered by pollinators than just one of them (Schoonhoven *et al*., 2005).

Different pollinators use different cues or combinations of cues. For example, syrphid flies often use petal size, where increase in petal size means more reward. Solitary bees however use the number of flowers, as primary cue to associate with the amount of rewards (Strauss *et al*., 1996).

Herbivory can change the quality and quantity of the rewards for pollinators, and they change the flower size, shape and number, which can change the pollinators preference and efficiency (Mothershead & Marquis, 2000). Therefore herbivory has potential to change the species composition of the pollinator community that are visiting a plant (Strauss *et al*., 1996).

Herbivory is believed to reduce the amount of nectar, and the quality of nectar in means of increase of secondary compounds and decrease in sugar content (Mutikainen & Delph,1996). However, it is also found that nectar with a high alkaloid concentration, has a higher sugar content, compared to nectar with a lower alkaloid concentration (Geagar *et al*., 2007). These (toxic) secondary compounds can act as deterrents for both herbivores and pollinators. They deter unwanted pollinators, like nectar thieves and robbers and inefficient pollinators, which are not adapted to the secondary compounds (Rhoades & Bergdahl, 1981; Adler, 2000; Liu *et al*., 2007). On the other hand, more tolerant pollinators are more efficient in transferring pollen (Singaravelan *et al*., 2005). Lepidopteran are known to be deterred by secondary compounds (Landolt & Lenczewski, 1993). Secondary compounds also reduce microbial degradation of the nectar (Adler & Irwin, 2005). Furthermore, the secondary compounds nicotine and caffeine may be addictive for insects, making them sort of dependent of the plant (Singaravelan *et al*., 2005).

Bees seem to be more adaptive to secondary compounds in nectar than other insects. Detzel & Wink (1993) showed that secondary compounds are common in plants pollinated primarily by bees, not in plants pollinated by butterflies. Thereby, Gegear *et al*. (2007) showed that nectar with high alkaloid concentration had higher sugar concentration. Carbohydrates mask the unpleasant taste of some secondary compounds, and bees act like they are balancing economic gains against costs (Liu *et al*., 2007). The pollen are rich of proteins and provide food for larvae of honey bees, and are often advertised by carotenoids, flavonoids or volatiles (Detzel & Wink, 1993).

Herbivory also affects pollinators by damaging reproductive tissue and/or floral characters to attract pollinators (Gegear *et al*., 2007). Bees more often visit perfect symmetric flowers, compared to those with symmetry imperfection, because it is thought perfect symmetry flowers produce more nectar (Schoonhoven *et al*., 2005).

1.8 Research

Together, herbivores and pollinators can affect the selective pressure in the evolution of plant responses and traits (Herrera *et al*., 2000; Mothershead & Marquis, 2000; Adler *et al*., 2001). Herbivores affect plant fitness by reducing its resources and damaging reproductive tissue (Strauss & Agrawal,1999), while plants try to prevent this, by evolving several defensive strategies. Pollinators influence plant fitness by transferring pollen from one plant to another, and thereby influencing its reproduction. Therefore, plants try to optimise pollen transfer, and will evolve strategies to attract effective pollinators.

In this research, the effects of herbivory on pollinators will be investigated. This will be done in terms of nectar quality and quantity, and the behaviour of pollinators. *Brassica nigra* will be used as plant species, and *Pieris brassicae* caterpillars as herbivores. The pollinators used will be *Apis mellifera*, *Pieris rapae* adults and *Episyrphus balteatus*.

Plants are affected by herbivores in terms of nectar an floral cues. The nectar, the component pollinators feed on, might change negatively by herbivores, by decreasing amount and sugar content, and increasing secondary compounds. Thereby floral cues, which pollinators use to determine which flower it will feed on, may also be changed by herbivory (Gegear *et al*., 2007). The flower display can be smaller, and the number of open flowers can decrease. This could negatively influence the pollinator behaviour, and they might avoid these flowers. The questions addressed to this subject will be:

1) *How is the quality and quantity of nectar influenced by foliar herbivores?*
2) *How do foliar herbivores influence the visitation and duration of visitation of different pollinators?*

The secondary compounds, glucosinolates are known to be produced by plants of the Brassicacea family, when herbivores feed on them. These mustard oil glycosides, are broken down to release volatile defensive substances (Taiz & Zeiger, 2002). The question addressed to this subject will be:

1) A. *Does nectar* of B. nigra *contain glucosinolates?*
   B. *Does the amount of nectar decrease due to herbivory?*

Different pollinators are used in this study, because it is known that different pollinators can act different to floral cues and change in nectar quality and quantity (Strauss *et al*., 1996).

2) *A. Do the number and of visits of* Pieris rapae *butterflies decrease due tot herbivory?*
   B. Do the number and of visits of Apis mellifera *decrease due tot herbivory?*
   C. Do the number and of visits of Episyrphus balteatus *decrease due tot herbivory?*

Secondary compounds in floral nectar can deter inefficient pollinators. Butterflies are known to be deterred by these (Landolt & Lenczewski, 1993). This could mean plants are trying to

deter them because they are inefficient pollinators, or that they are deterred not on purpose. Butterflies have long tongues to feed on nectar, and some plants are adapted to that by elongated flowers, to maximize the contact of the butterfly with the pollen. However, the shorter flowers of non adaptive plant species may not be pollinated by butterflies at all, while they are feeding, because the body never contacts the pollen. The question remains if butterflies in some cases are effective pollinators, or more nectar robbers. In this research the specialist herbivores *Pieris rapae* butterflies are used for the pollinator. The question addressed to this subject will be:

3) *Are* Pieris rapae *butterflies effective pollinators for* Brassica nigra *plants?*

2. Material and methods

2.1.1 Study organisms

***Brassica nigra* (black mustard)**

The plants used in the experiments were *B. nigra* (Black mustard). The experiments were performed with +/- 7 week old plants, grown in a greenhouse at 22 ± 2 ºC, 50-70% r.h. under a L16:D8 photoperiod. During the experiments, the plants were kept under the same conditions in an other greenhouse compartment. The plants were grown from seeds collected in the field in 2005 from *B. nigra* accession CGN06619 open-pollinated plants.

***Pieris brassicae* (large white cabbage butterfly)**

Pieris brassicae was used as a herbivore in the experiments. The butterflies were reared on Brussels sprouts (*Brassica oleracea* var. *gemmifera*) at 22 ± 2 ºC, 50-70% r.h. under a L16:D8 photoperiod.

Pollinators

Three different pollinator species were used during the experiments; marmalade flies (*Episyrphus balteatus*), honey bees (*Apis mellifera*) and small cabbage white butterflies (*Pieris rapae*).

***Episyrphus balteatus* (marmalade fly)**

The syrphid fly species used in this research was *E. balteatus.* They were ordered at *Koppert Biological Systems* (www.koppert.com), a company specialised in biological pollination and pest management. This product was called SYRPHIDEND, and came in packages with 50 pupae glued on a card.

The larva of *E. balteatus* are important biological agents of aphids. Adults are known to prefer yellow and small flowers, and are able to discriminate between different qualities of rewards. The adult flies feed on pollen and nectar (Sutherland *et al*., 1999). The pupae were hung in a cage, allowing the flies to hatch. During the experiment weeks, the flies were fed on 10% sugar water, provided in artificial yellow flowers and pollen collected from *B. nigra* plants.

***Pieris rapae* (small cabbage white butterfly)**

P. rapae was used as one of the pollinators in the experiments. The butterflies were reared on Brussels sprouts (*Brassica oleracea* var. *gemmifera*) at 22 ± 2 ºC, 50-70% r.h. under a L16:D8 photoperiod. They were fed on 10 % sugar water in a yellow bottle cap and provided with a Brussel sprout plant for oviposition, during the weeks in which the choice experiment were performed. The butterflies were kept in a cage in the greenhouse compartment, before using them in the experiments.

***Apis mellifera* (honey bee)**

A small *A. mellifera* colony (+/- 300 bees) was provided by commercial beekeepers from *Inbuzz* (www.inbuzz.nl), and consisted of three frames with brood of all stages plus the laying queen. The hive with bees was held in a gauze tent within a greenhouse compartment for three weeks, to prevent the bees from flying around in the greenhouse compartment. During this time, the bees were fed on 10% sugar water (provided in artificial yellow flowers), water and were provided *B. nigra* plants to feed on the flowers, which were previously used for the pollinator choice experiments.

2.1.2 Herbivore treatment

P. brassicae caterpillars were used to examine the effects of herbivory on *B. nigra*, in terms of nectar production and pollinator attraction. Real caterpillars were used, because elicitors from oral and oviduct secretions play an important role in the induction of the plant defence pathway (Dicke *et al*., 2002).

For the herbivore treatment, third instar *P. brassicae* caterpillars were used. The *B. nigra* plants received 50 caterpillars per leaf, two leaves per plant. This amount of caterpillars was used to cause a sufficient amount of damage, to create a defence response in the plant. One batch of eggs laid by *P. brassicae* consists of approximately 50 eggs, which means that the amount of caterpillars used per plant, are comparable to two egg batches. The caterpillars were encaged in small cages (modified petridishes) to prevent migration of the caterpillars to the flowers (figure 2.1.2).

Figure 2.1.2: The small cages the caterpillars were kept in to prevent migration.

These cages were repositioned every day during the treatment, because the caterpillars within a cage consumed a whole leaf within a day. There were three herbivore treatments; 24, 48 and 72 hour periods of feeding by *P. brassicae*, to follow the change in defence over time.

The control plants received no further treatments and were held under the same conditions as the herbivore treatment plants.

2.2 Nectar experiments

2.2.1 Nectar extraction

To examine whether the secretion of nectar by *B. nigra* changed due to herbivory, nectar was collected. The amount of nectar of both herbivore infested and control plants was measured, and compared with each other. The nectar extraction took place in the morning around nine o'clock, since the nectar production is known to be the highest in the morning. One hour before the nectar extraction, a humidifier (Defensor 3001) was turned on, to increase the air humidity, and thereby the amount of nectar. The nectar extraction took place approximately 24, 48 and 72 hours after the herbivore treatment.

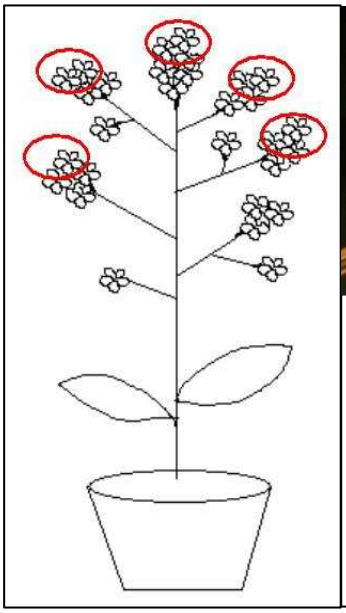

Figure 2.2.1: Nectar extraction. (left) Nectar was extracted from the first five successive flowers at the top of a flowering branch, from five branches: the top, second, third, fourth and fifth branch. (above) Nectar was extracted with a capillary tube.

The flowers of *B. nigra* are small, and therefore capillary tubes were used to collect the nectar (figure 2.2.1). These capillary tubes were made pointy, to maximize the nectar uptake. This was preformed by using a heater machine (NARISHE model PB) set on 100 ^{o}C, which heated the end of a 5 µl capillary tube, elongating it with a weight of 90 g, thereby creating a pointy end which has been proven very useful for the uptake of nectar (Bruinsma *et al*, 2008).

The nectar was extracted from 25 flowers per plant, from the first five successive flowers at the top of a flowering branch, from five branches: the top, second, third, fourth and fifth branch (figure 2.2.1).

2.2.2 Determining nectar amount

After the extraction of nectar, the amount of nectar collected was measured using a measuring rod. The number of measured centimetres was converted to µl's (1,5 cm= 1 µl). After measuring the amount of nectar, the nectar was put in an Eppendorf tube, using a small plastic tube to blow the nectar out of the capillary tube. Afterwards the Eppendorf tubes were stored in a refrigerator by –20 ^{o}C, waiting for further analyses.

In total, 122 nectar samples were collected, 62 from herbivore treated plants and 60 from control plants.

2.2.3 Nectar analyses

To examine whether the quality of nectar changed due to herbivory the presence of glucosinolates were analysed. The glucosinolate analyses were done at the NIOO (Netherlands Institute of Ecology).

2.3 Pollinator choice experiments

2.3.1 Pollinator choice experiment

The effect of herbivory on the attraction of pollinators by *B. nigra*, was examined by performing pollinator choice experiments. The plants were tested in a two-choice experiment with one control and one herbivore-infested plant, to record the flower visiting behaviour of the pollinators. Different pollinators were used, because it is known that different pollinators can act different to floral cues and change in nectar quality and quantity (Strauss *et al*., 1996). The pollinators used for these experiments were; the adults of *A. mellifera*, *E. balteatus* and *P*. *rapae*.

The experiments were performed in a gauze tent, with a ground surface of 293 cm x 200 cm and a height of 230 cm, to prevent the pollinators from flying loose in the greenhouse compartment. Two plants were put in the tent, one control plant and one herbivore treated plant (figure 2.3.1). The position of the treated plants was changed after every experiment, to prevent that the position of the plants influenced the behaviour of the pollinators. For every experiment, new plants were used to prevent pollinators would use cues left by pollinators in previous experiments.

Before the experiments, the total number of open flowers of the plants used was counted, to equalize the number of open flowers of the two plants (herbivore treated and control plant) used in the same experiment, because it is known that the number of open flowers can also affect the attraction of the pollinators (Straus *et al*., 1996).

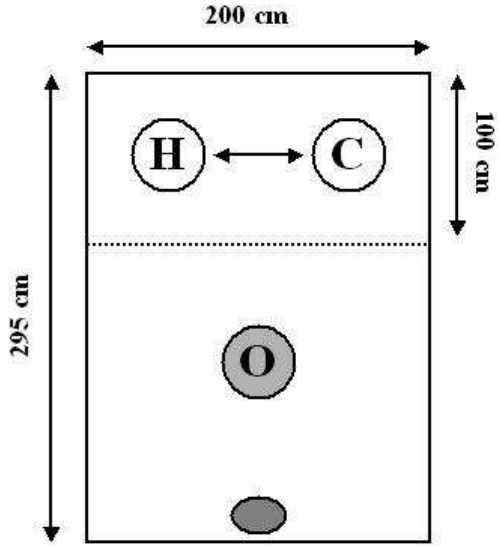

Figure 2.3.1: Gauze tent in which pollinator choice experiments were performed. H= herbivore treated plant, C = control plant, O= place of observation, dark grey oval = position of camera and the arrow = the positions of the herbivore treated and control plants was often changed. One third of the compartment was used for the pollinator choice experiment with the butterflies.

In all the pollinator choice experiments the number of visitations and the duration of the visitations was recorded, to examine whether they differed between control and herbivore infested plants. The number of visitations was recorded as the total number of pollinators visiting a plant during a time span of 20 minutes (*E. balteatus* and *P. rapae*) or the average number of pollinators on a plant at different time intervals during 9 minutes (*A. mellifera*). The duration of visitation was recorded as the time spent on one flower.

This experiment was repeated 36 times, with 72 plants, 36 herbivore treated and 36 control plants *for E. balteatus* and *P. rapae*, and per herbivore treatment interval (24, 48 and 72 hours) 11 experiments were preformed. For *A. mellifera* the experiments were repeated 30 times, with 60 plants, 30 herbivore treated and 30 control plants, and per herbivore treatment interval (24, 48 and 72 hours) 10 experiments were preformed.

The pollinator choice experiments of *P. rapae* and *E. balteatus*, were preformed at the same day and with the same plants as the nectar extraction experiments, a few hours after the collection of nectar.

2.3.2 *Episyrphus balteatus* choice experiments

For these pollinator choice experiments, eight syrphid flies were used per series of two plants, one control and one herbivore treated plant. The syrphid flies were used at least one

day after hatching from the pupae and were starved for 24 hours before the experiment. During their starvation they were fed on water only.

The eight syrphid flies were marked individually using fluorescent powder (FIESTA daylight fluorescent colours), to distinguish and follow the different individuals. The fluorescent powder was applied on the syrphid flies thorax by using a syringe needle (figure 2.3.2). This was done one hour before the experiments started.

Figure 2.3.2: Marking of syrphid fly with fluorescent powder.

The choice experiments started at 13:30. The eight syrphid flies were released in the middle of the tent, giving them one minute to adjust to their surrounding before starting the experiment. The two plants were followed at the same time for 20 minutes, recording the number of syrphid flies visiting the flowers and leaves and the duration of the flower visitations with an handheld computer (Psion Workabout), programmed with The Observer (version 4.1, Noldus Information Technology, Wageningen, The Netherlands). The behaviour of individual syrphid flies was recorded, in terms of plants visited and the kind of nourishment the took (feeding on nectar or pollen).

2.3.3 *Pieris rapae* choice experiments

For these pollinator choice experiments, eight *P. rapae* butterflies were used per series of two plants, one control and one herbivore treated plant. The butterflies were used at least two days after hatching from the pupae, and were starved over night before the experiment, provided only with water. A Brussel sprout was provided for oviposition, to prevent the butterflies from ovipositing during the experiments.

The eight butterflies were marked by putting a number on both lower wings using a waterproof marker, to distinguish and follow the different individuals (figure 2.3.3).

Figure 2.3.3: Marked butterfly with waterproof marker.

The choice experiments started at 10:30, since it is known that butterflies are most active during the morning. The two plants (control and herbivore infested) put in the gauze tent, were put on boxes, so that the flowers would reach the top of the tent. The part of the tent in which the plants were positioned, was enclosed by a gauze cloth, creating a space within the tent of 100 cm x 200 cm and 230 cm in height, used to perform the experiments is (figure 2.3.1). During a pilot study this appeared to be the best set-up to allow flower visitation by the butterflies.

The eight butterflies were released in the tent, allowing them five minutes to adjust to their surrounding before starting the experiment. The two plants were followed at the same time for 20 minutes, recording the number of butterflies visiting the flowers and the duration of those visitation with a handheld computer (Psion Workabout), programmed with *The Observer*. The behaviour of the individual butterflies was recorded, in terms of plants visited and oviposition.

During the third week of the pollinator choice experiments, trips appeared on the plants. They were abundant on the plants during eight series of experiments.

2.3.4 *Apis mellifera* choice experiments

For these pollinator choice experiments, a small colony of a few hundred bees was used for all the choice experiments. The colony was used per series of two plants, one control and one herbivore treated plant.

The choice experiments started at 13:30. After the plants were put in the tent, the bees had five minutes to adjust tot the changes in their surrounding. The number of visitations and the duration of visitation was recorded on camera (Panasonic, NV-GS230EG/EF/EK). Both plants were followed at the same time for 10 minutes.

The movies were analysed by burning them on a DVD and watching them with Windows Media player. The number of visitations was measured by counting all the bees present on a plant at time intervals of 30 seconds. For the statistical analysis, the average of the number of bees, for all the intervals during 9 minutes, was used. The duration of visitation was measured by recording all the visitations of all the bees during one minute of the movie, using a stopwatch.

2.3.5 *Pieris rapae* effectiveness as pollinator

The pollination effectiveness of *P. rapae* as pollinator for *B. nigra* was examined by measuring the number of seeds set in plants visited by butterflies, comparing it with the seeds set in control plants. For these experiments, half of the plants were put in the gauze tent with 10 male butterflies for 48 hours, to give the butterflies enough time to visit different flowers. Male butterflies were used instead of females, because female butterflies oviposit on the plants. If eggs are lead on a plant, they are difficult to discover, and when they are not removed, caterpillars would hatch. These caterpillars also feed on flowers, which would influence this experiment.

The other half of the plants were kept outside the gauze tent in the same greenhouse compartment. Afterwards the plants were stored in the same greenhouse compartment for six weeks giving them time to set seed. In the fourth week the plants were packed in plastic to prevent seed loss. In the fifth week aphids appeared on the plants. The seeds were

collected by cutting of the lower half of the plant and then gently squeezing all the seeds out of the upper half of the plant. The collected seeds were separated from the plant material using a sieve. The number of seeds per plant were counted by hand.

In total 28 plants were used, 14 control plants and 14 “pollinated” plants.

2.4 Statistical analyses

To test whether the amount of nectar differed between the control and herbivore infested plants, a General linear model (GLM) was performed (SPSS 15.0). The data set was first transformed (ln(a+0.001)) to create a normal distribution. Two fixed factors were used; treatment and day, because there was a big variation in the amount of nectar collected, depending on the day.

A Wilcoxon test was performed, to test for differences between the number of visits of *E. balteatus*, *P. rapae* and *A. mellifera* between the control en herbivore infested plants.

To test whether the duration of visits of *E. balteatus*, *P. rapae* and *A. mellifera* differed between the control en herbivore infested plants, the Mann Whitney U test was used.

For correlation analyses, the Spearman rank test was used.

3 Results

3.1 Nectar experiments

A large variation was found in the amount of nectar depending on the day it was collected, probably due to variations in air humidity in the greenhouse compartment. Therefore, the days on which the nectar was collected, were included in the statistical analyses.

The amount of nectar, collected from control and herbivore infested plants, differed significantly based on the treatment (*P*= 0.047, GLM) and on the day (*P*< 0.001, GLM). The combination of treatment and day did not have an influence on the differences in nectar amount between the two plant treatments (*P*= 0.541, GLM) (figure 3.1.1).

Within all the treatment durations, the amount of nectar only differed significantly based on the day (C24-H24 *P*< 0.000, C48-H48 *P*< 0.001, C72-H72 *P*< 0.001, GLM). The treatment itself and the combination of treatment and day had no significant effect (treatment; C24-H24 *P*= 0.120, C48-H48 *P*= 0.247, C72-H72 *P*= 0.163, GLM) (treatment*day; C24-H24 *P*= 0.766, C48-H48 *P*= 0.831, C72-H72 *P*= 0.437, GLM) (figure 3.1.2). Although the nectar volume tended to be lower in the flowers of herbivore-infested plants.

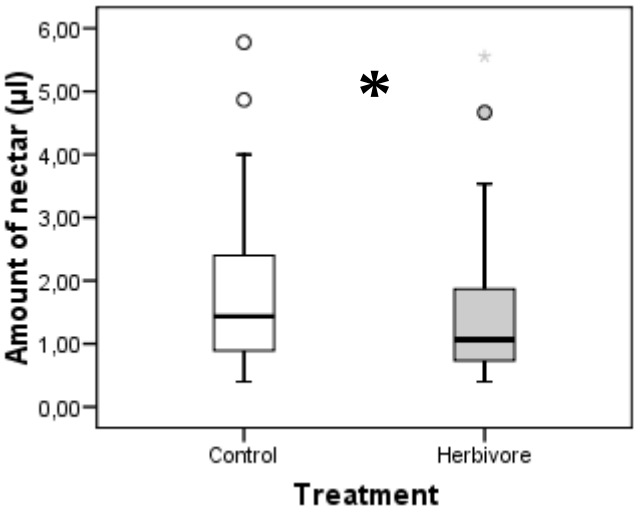

Figure 3.1.1: Boxplot of the amount of nectar of control- and herbivore infested plants (N=66), *= (*P*> 0.05).

- ***treatment* (df= 1, F= 4.079, *P*= 0.047, GLM)**
- ***day* (df= 24, F= 9.212, *P*< 0.001, GLM)**
- ***treatment*day* (df=24, F= 0.948, *P*= 0.541, GLM)**

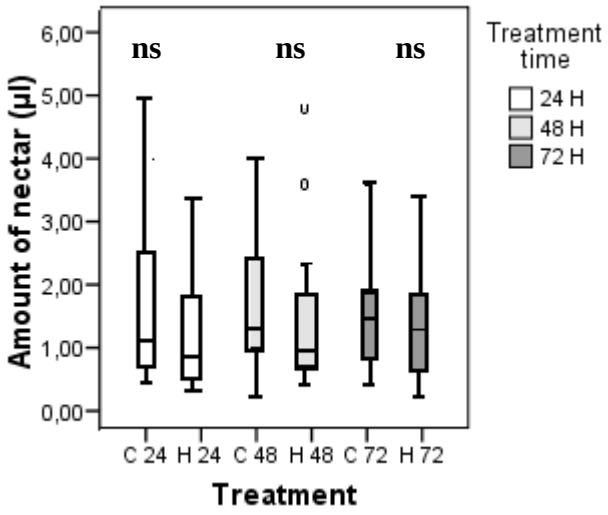

Figure 3.1.2: Boxplot of the amount of nectar of control and herbivore infested plants of different treatment times. C= control, H= herbivore, 24= 24 hour treatment, 48= 48 hour treatment and 72 = 72 hour treatment, ns= not significant.

- **<u>C24-H24</u> (N= 22)**

***treatment* (df=1, F= 2.624, *P*= 0.120, GLM)**
***day* (df=10, F= 14.476, *P*< 0.001, GLM)**
***treatment*day* (df= 10, F= 0.639, *P*= 0.766, GLM)**

- **<u>C48-H48</u> (N= 22)**

***treatment* (df= 1, F= 1.413, *P*= 0.247, GLM)**
***day* (df= 10, F= 5.561, *P* < 0.001, GLM)**
***treatment*day* (df= 9, F= 0.539, *P*= 0.831, GLM)**

- **<u>C72-H72</u> (N= 22)**

***treatment* (df= 1, F= 2.081, *P*= 0.163, GLM)**
***day* (df= 8, F= 12.820, *P*< 0.001, GLM)**
***treatment*day* (df= 8, F= 1.041, *P*= 0.437, GLM)**

3.2 Pollinator choice experiments

Episyrphus balteatus

This study showed a significant difference in the number of visits of *E. balteatus* between the control and herbivore infested plants (*P*< 0.001, Wilcoxon test) (figure 3.2.1). The number of visits also differed significantly for the 24 hour treatment, between control and herbivore infested plants (C24-H24 *P*= 0.014, Wilcoxon test). Although the other two treatment durations did not differ significantly, the syrphid flies tended to visit herbivore infested plants less often compared to the control plants (C48-H48 *P*= 0.070, C72-H72 *P*= 0.072, Wilcoxon test) (figure 3.2.2).

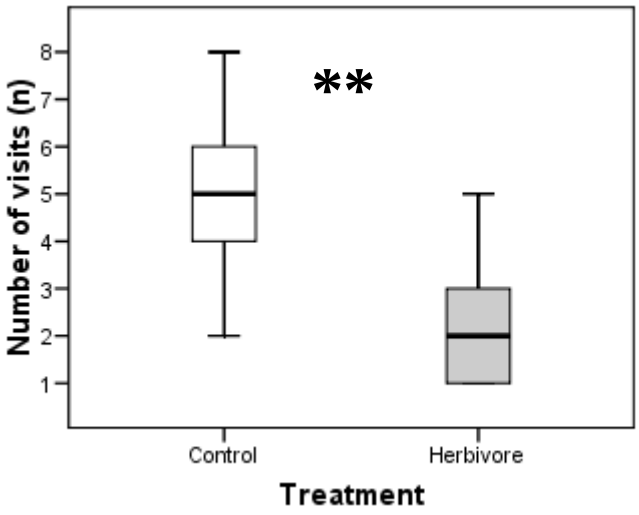

Figure 3.2.1: Boxplots showing the number of visits of *Episyrphus balteatus* on control and herbivore infested plants, **= (*P* < 0.005) (N= 36, *P*= 0.01, Wilcoxon test).

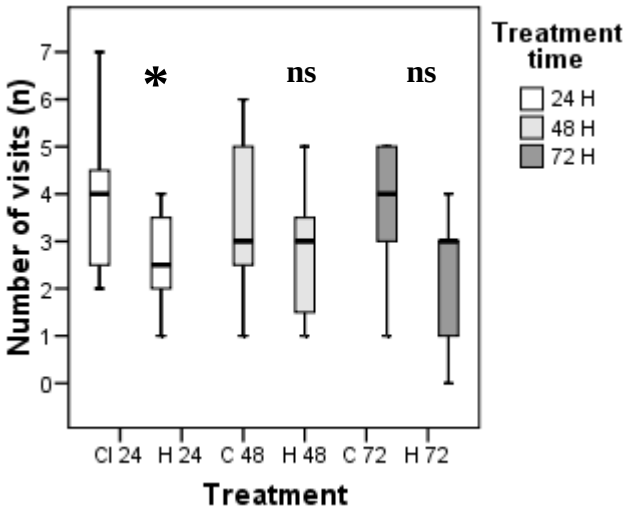

Figure 3.2.2: Boxplots showing the number of visits of *Episyrphus balteatus* on control and herbivore infested plants of different treatment times. C= control, H= herbivore, 24= 24 hour treatment, 48= 48 hour treatment and 72 = 72 hour treatment, *= (*P*< 0.05), ns= not significant.
(C24-H24 N= 12, *P*= 0.014, C48-H48 N= 12, *P*= 0.070, C72-H72 N= 12, *P*= 0.072, Wilcoxon test)

The durations of visits of *E. balteatus*, differed significantly, between the control and herbivore infested plants (*P*< 0.001 Mann Whitney U test). The herbivore infested plants were visited shorter compared to the control plants (figure 3.2.3). The durations of visits also differed for al the treatment durations (C24-H24 *P*= 0.001, C48-H48 *P*= 0.016, C72-H72 *P*< 0.001, Mann Whitney U test) (figure 3.2.4).

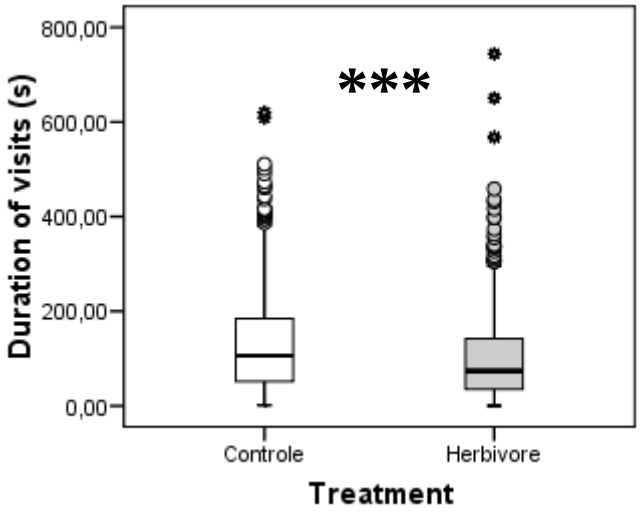

Figure 3.2.3: Boxplots showing the duration of visits of *Episyrphus balteatus* on control and herbivore infested plants, *=(*P*< 0.001) (N= 36, *P*< 0.001, Mann Whitney U test).**

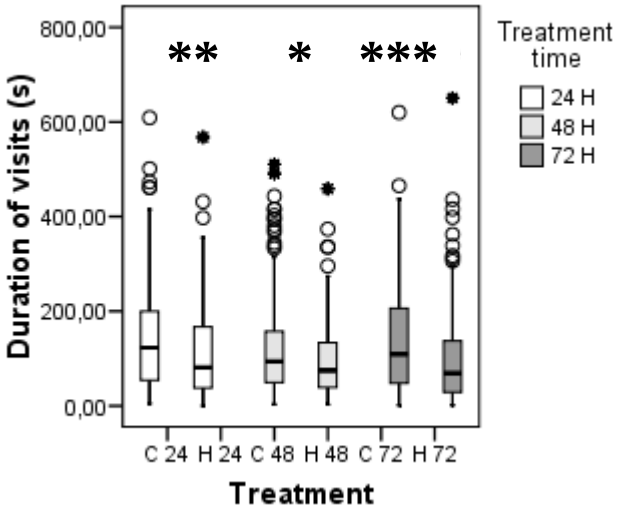

Figure 3.2.4: Boxplots showing the duration of visits of *Episyrphus balteatus* on control and herbivore infested plants of different treatment times. C= control, H= herbivore, 24= 24 hour treatment, 48= 48 hour treatment and 72 = 72 hour treatment, *= (*P*< 0.05), **= (*P*< 0.005), *=(*P*< 0.001) C24-H24 N= 12, *P*= 0.001, C48-H48 N= 12, *P*= 0.016, C72-H72 N= 12, *P*< 0.001, Mann Whitney U test).**

E. balteatus tended to visit leaves of herbivore infested plants more often compared to leaves of control plants. Leaves of herbivore infested plants received 24 individuals, while control plant leaves were only visited by 14 individuals (Appendix 6.1).

In only four of the 26 series, some *E. balteatus* individuals were actually feeding on nectar. In the other series they only consumed pollen.

Pieris rapae

A significant difference was found in the number of visits by *P. rapae* between the control and herbivore infested plants (*P*= 0.001, Wilcoxon test). The herbivore infested plants received less visits than the control plants (figure 3.2.5). The butterflies also showed a significant difference in number of visits in all the treatment durations (C24-H24 *P*= 0.003, C48-H48 *P*= 0.010, C72-H72 *P*= 0.003, Wilcoxon test) (figure 3.2.6).

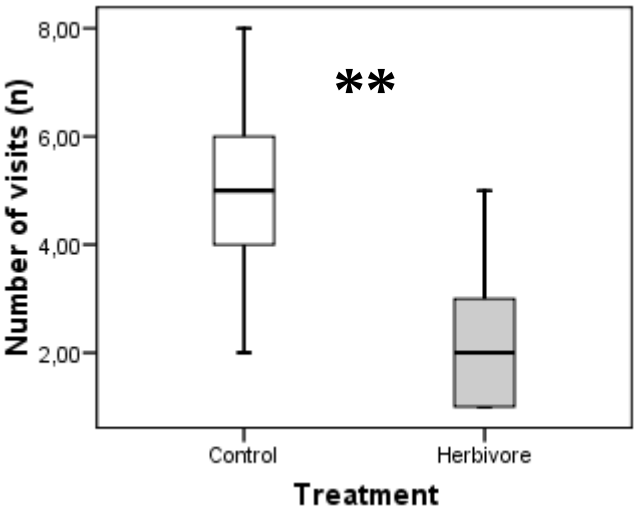

Figure 3.2.5: Boxplots showing the number of visits of *Pieris rapae* on control and herbivore infested plants, **= (*P*< 0.005) (N= 36, *P*= 0.001, Wilcoxon test).

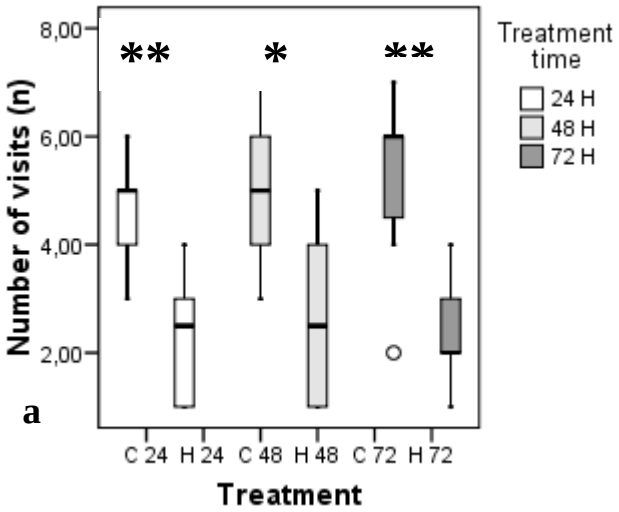

Figure 3.2.6: Boxplots showing the number of visits of *Pieris rapae* on control and herbivore infested plants of different treatment times, C= control, H= herbivore, 24= 24 hour treatment, 48= 48 hour treatment and 72 = 72 hour treatment , *= (*P*> 0.05), **= (*P*< 0.005), (C24-H24 N= 12, *P*= 0.003, C48-H48 N= 12, *P*= 0.010, C72-H72 N= 12, *P*= 0.003, Wilcoxon test).

P. rapae showed a significant difference in duration of visits, between the control and herbivore infested plants (*P*< 0.001 Mann Whitney U test) (figure 3.2.7). Within all the different treatment durations, the duration of visits differed significantly between the control and herbivore infested plants (C24-H24 *P*< 0.001, C48-H48 *P*< 0.001, C72-H72 *P*< 0.001, Mann Whitney U test) (figure 3.2.8).

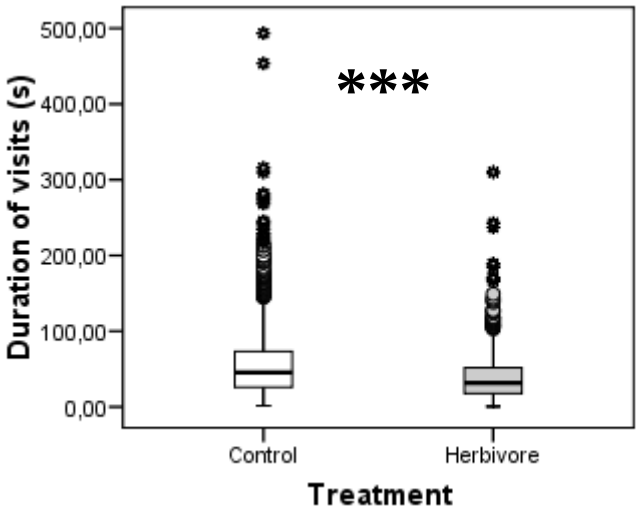

Figure 3.2.7: Boxplots showing the duration of visits of *Pieris rapae* on control and herbivore infested plants, *=(*P*< 0.001) (N= 36, *P*< 0.000, Mann Whitney U test).**

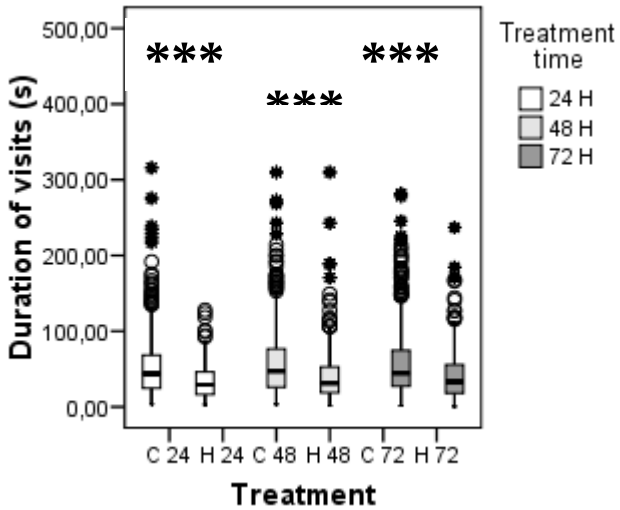

Figure 3.2.8: Boxplots showing the duration of visits of *Pieris rapae* on control and herbivore infested plants of different treatment times. C= control, H= herbivore, 24= 24 hour treatment, 48= 48 hour treatment and 72 = 72 hour treatment., *=(*P*< 0.001) (C24-H24 N= 12, *P*< 0.001, C48-H48 N= 12, *P*< 0.001, C72-H72 N= 12, *P*< 0.001, Mann Whitney U test).**

The leaves of herbivore infested plants tended to be more often visited by *P. rapae* individuals, than the leaves of control plants. The control plant leaves received 10 visits, while the herbivore infested plant leaves received 22 visits (Appendix 6.2). However, the ovipositioning by *P. rapae* individuals occurred on both herbivore infested and control plants (Appendix 6.3).

During the third week of the pollinator choice experiments, trips appeared on the plants of 8 series of experiments. The number of visits did not differ between series with and without trips (H-H with trips $P= 0.187$, C-C with trips $P= 0.734$, Mann Whitney U test). The duration of visits did differ between series with and without trips (H-H with trips $P< 0.001$, C-C with trips $P< 0.037$, Mann Whitney U test) (Appendix 6.4).

Apis mellifera

A. mellifera showed no significant difference in the number of visits between the control and herbivore infested plants ($P= 0.350$ Wilcoxon test) (figure 3.2.9). They also showed no significant difference in number of visits within the same treatment duration between the control and herbivore infested plants (C24-H24 $P= 0.241$, C48-H48 $P= 0.575$, C72-H72 $P= 0.610$, Wilcoxon test). The bees showed no clear tendency towards differences in number of visits (figure 3.2.10).

It is known that some bee species use the number of flowers, as primary cue to associate with the amount of rewards (Strauss *et al*., 1996). However, the number of visits did not correlate with the number of flowers of the plants visited by *A*. *mellifera* ($P= 0.100$, Spearman rank test) (Appendix 6.5).

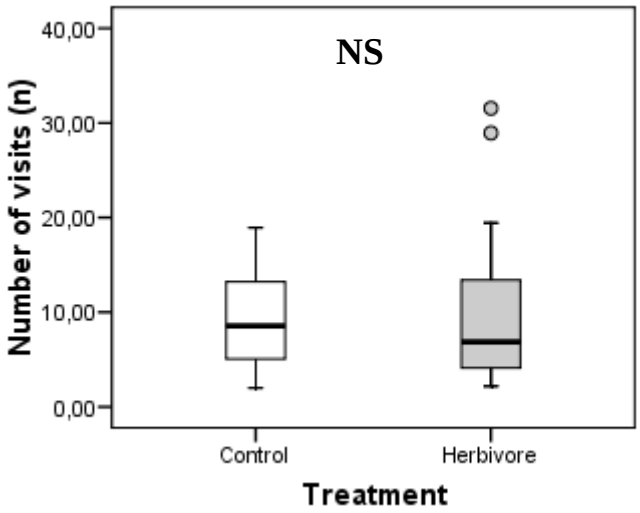

Figure 3.2.9: Boxplots showing the number of visits of *Apis mellifera* on control and herbivore infested plants, NS= not significant (N= 30, *P*= 0.355), Wilcoxon test)

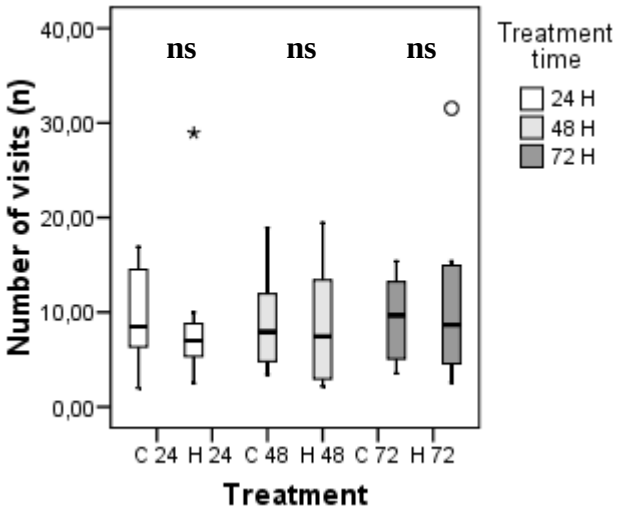

Figure 3.2.10: Boxplots showing the number of visits of *Apis mellifera* on control and herbivore infested plants of different treatment times. C= control, H= herbivore, 24= 24 hour treatment, 48= 48 hour treatment and 72 = 72 hour treatment, ns= not significant (C24-H24 N= 10, *P*= 0.241, C48-H48 N= 10, *P*= 0.575 , C72-H72 N= 10 *P*= 0.610. Wilcoxon test).

The duration of visits of *A. mellifera* differed significantly between control and herbivore infested plants (*P*< 0.001 Mann Whitney U test) (figure 3.2.11). The different treatment durations also showed a significant difference in the duration of visits (C24-H24 *P*< 0.001, C48-H48 *P*< 0.001, C72-H72 *P*< 0.001, Mann Whitney U test) (figure 3.2.12).

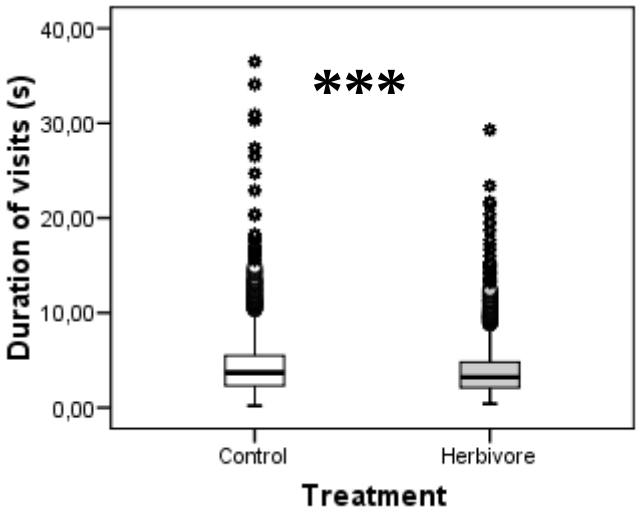

Figure 3.2.11: Boxplots showing the duration of visits of *Apis mellifera* on control and herbivore infested plants, *=(*P*< 0.001) (N= 30, *P*< 0.000, Mann Whitney U test).**

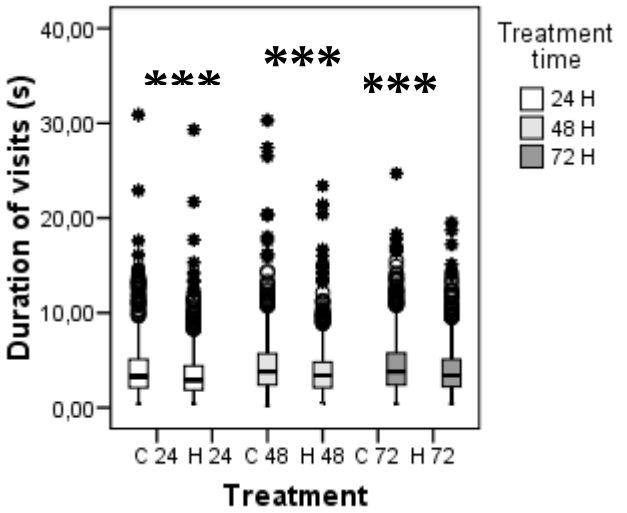

Figure 3.2.12: Boxplots showing the duration of visits of *Apis mellifera* on control and herbivore infested plants of different treatment times. C= control, H= herbivore, 24= 24 hour treatment, 48= 48 hour treatment and 72 = 72 hour treatment, *=(*P*< 0.001) (C24-H24 N= 10, *P*< 0.001, C48-H48 N= 10, *P*< 0.001 , C72-H72 N=10, *P*< 0.001, Mann Whitney U test).**

Correlation between duration of visits and nectar amount

The nectar extraction and pollinator choice experiments of *P. rapae* and *A. mellifera* were performed on the same plants, which gave the opportunity to determine whether there was a relationship between the amount of nectar and the duration of pollinator visits.

The amount of nectar did not correlate with the duration of visits of *P. rapae* (*P*= 0.242, Spearman rank test) (Appendix 6.6). The amount of nectar did also not correlate with the duration of visits of *A. mellifera* (*P*= 0.343, Spearman rank test) (Appendix 6.7).

3.3 Pollination effectiveness of *Pieris rapae*

A significant difference was found in number of seeds set between control plants and plants visited by *P. rapae* butterflies (*P*= 0.039, Mann Whitney U test) (figure 3.3). The plants visited by the *P. rapae* butterflies set more seed compared to the plants that did not receive any pollinator.

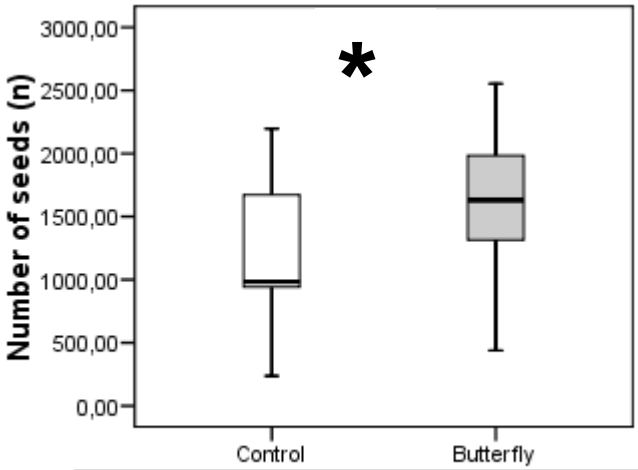

Figure 3.3: Boxplot of the number of seeds set by control plants and plants visited by butterflies of *Pieris rapae*, *= (*P*> 0.05) (N= 14, *P*= 0.039, Mann Whitney U test).

4 Discussion

Plant traits

The main effect of herbivory is the reduction of plant biomass, and thereby decreasing the photosynthetic area of the plant. This causes a decrease in resources left for the plant, thereby changing the allocation of these resources to different plant parts, and reducing plant growth (Mothershead & Marquis, 2000). Therefore many changes can take place in plant traits due to herbivory, like changes in flower number, flower size, pollen quality and quantity, nectar quality and quantity, volatiles, seed size and number (Strauss *et al*., 1996; Mutikainen & Delph, 1996; Quesada *et al*., 1995; Strauss & Murch, 2004; Arimura *et al*., 2005).
In this research the changes in nectar quantity and pollinator attraction were studied. It was shown that the amount of nectar decreased due to foliar herbivory. Strauss *et al*. (1996) also found that the nectar production was lower in damaged plants, but was extremely variable, and differences were only marginally significant. This high variability was also found in this study, probably due to the variability in air humidity from day to day.

Secondary metabolites are found in the nectar of certain plant species. In this study glucosinolates were present in the nectar of *B. nigra*. Several hypotheses have been proposed which could explain the ecological and evolutionary function of secondary compounds in nectar. Secondary compounds in nectar could function as deterrents for nectar thieves/robbers and inefficient pollinators (Rhoades & Bergdahl, 1981; Adler, 2000; Liu *et al*., 2007; Landolt & Lenczewski, 1993). They also might protect nectar from degrading carbohydrates by microbes (Hagler & Buchmann, 1993). However, the abundance of secondary compounds in nectar might be a consequence of production of secondary compounds in other tissue, having no adaptive function at all, because effective pollinators could be deterred (Singaravelan *et al*., 2005).

Syrphid flies

E. balteatus reacted to foliar herbivory, by visiting plants with caterpillars less often and with shorter durations of visits, than plants without herbivores. The reason for this behaviour is not clear. However, nectar can probably be excluded as the cause of this behaviour, because there was no correlation found between the duration of visits and the amount of nectar. During the pollinator choice experiments the syrphid flies primarily fed on pollen instead of nectar. The difference in the number and duration of visits of this species due to herbivory was therefore probably not caused by differences in nectar quality or/and quantity, but rather by differences in pollen quality or/and quantity. However, Sutherland *et al* (1999) found that *E. balteatus* did not discriminate between artificial flowers with increasing amounts of pollen present. Therefore, if they react to pollen, it is probably because of the quality of pollen

instead of the pollen quantity. On the other hand it is also reported that *E. balteatus* strongly reacts to differences in honey concentration, and were able to distinguish between different concentrations (Sutherland *et al*., 1999). Therefore they should also be able to distinguish between different sugar concentration in nectar.

To examine the reaction of *E. balteatus* to possible changes in nectar amount and quality due to herbivory, the proportion of syrphid flies eating nectar should be maximized. It would therefore be better to use males alone, because they collect more nectar then females. This is often observed during field experiments. Both males and females need pollen for the gametogenesis, but females also ingest pollen for the yolk deposition in the eggs. Males, on the other hand, have a higher energy requirement because of the hovering behaviour, and therefore collect more nectar (Sutherland *et al*., 1999).

Although the syrphid flies did not consume a lot of nectar, they did respond to foliar herbivory. Therefore, it would be interesting to examine whether the amount and quality of pollen is affected by foliar herbivory. There are studies that imply that the amount and quality of pollen produced by plants, decrease due to leaf damage (Quesada *et al*., 1995; Strauss *et al*., 1999; Mutikainen & Delph, 1996). The quality of pollen was measured by examining the likeliness to sire seed, it is known that the pollen of the plants with leaf damage were less likely tot sire seed than the pollen of undamaged plants (Quesada *et al*., 1995; Mutikainen & Delph, 1996). For this research it would be more relevant to examine the quality of pollen in means of quality as reward for pollinator, instead of quality for plant reproduction, although they might be coupled. On the other hand, the pollen may not have been affected by herbivory during this experiment. They can only be affected before or while maturation of pollen, not afterwards (Mutikainen & Delph, 1996).

Butterflies

The *P. rapae* butterflies responded to foliar herbivory with a decrease in number of visits and a shorter duration of visitation, when compared to the control plants. The duration of visits did not correlate with the amount of nectar. So, it seems that the amount of nectar does not determine the duration of visits. Other aspects, like nectar quality or flower volatiles, could have caused the preference for the flowers of the control plants against the flowers of the herbivore infested plant.

The choice of *P. rapae* females for a certain plant could also be influenced by plant cues used for oviposition. Bruinsma *et al* (2008) found that female *P. rapae* avoided plants treated with jasmonic acid. Although there were butterflies that oviposited during the pollinator choice experiments, they did not show a preference for control plants over herbivore infested plants (Appendix 6.4). However, floral volatiles can also affect the foraging behaviour of *P. rapae* (Honda *et al*., 1998). Female *P. rapae* visit plants for both foraging and

ovipositioning, and therefore might use different cues for them which can influence the choice of a female for a certain plant.

It is known that certain herbivores, which are often specialist, can be attracted to higher concentrations of secondary compounds which can serve as oviposition and foraging stimulants. The negative effects of these compounds on the development of the adapted herbivores are often quite low (Strauss *et al*., 1999). If herbivore infested *B. nigra* plants would contain higher concentrations of glucosinolates, it would be expected that *P. rapae* would be attracted to the herbivore infested plants instead of the control plants. However this was not found in this experiment.

Bees

The number of visits of *A. mellifera* did not change due to foliar herbivory, while they did with *E. balteatus* and *P. rapae. Apis mellifera* did not respond in a similar way to foliar herbivory as *E. balteatus* and *P. rapae*. Strauss *et al* (1996) already mentioned that different pollinator groups use different cues, and are therefore differently affected by changes in floral characters due to herbivory. They also found that some bee species use the number of flowers as primary cue to associate with the amount of rewards. However, the number of visits did not correlate with the number of open flowers, in this study. The two plants used for one pollinator choice experiment were chosen on equal number of open flowers, to prevent that numbers of open flowers affected the choice of the pollinators. Therefore the differences in number of flowers of the plants used in one experiment were minimal. This might be a reason why there was no correlation found between the number of visits and the number of open flowers. Lehtilä and Strauss (1997) found that there was no difference in number of visits of native bees between damaged and undamaged plants when the number of open flowers was controlled.

The number of visits of *A. mellifera* did not correlate with the amount of nectar. But it is known that bees use the shape and size of flowers to associate with a bigger nectar amount (Schoonhoven *et al*., 2005). The size and shape of the flowers of control plants and herbivore plants in this experiment did probably not differ. And if the shape differed, this was certainly not due to the herbivore treatment, because the caterpillars were not introduced at the right time to change flower shape/size and number. To affect these traits they would need to be introduced at an early stage of development, when flowers are not present yet and still have to develop (Lehtilä & Strauss, 1997; Strauss, 1996).

There might be another reason why a difference in number of visits was not found. The method to estimate the number of visits for *A. mellifera* differed from the method used for *E. balteatus* and *P. rapae*. For *E. balteatus* and *P. rapae* the actual number of visits during the 20 minutes of the pollinator choice experiment was used, while for *A. mellifera* an

average of number of bees present on a plant at a certain time was used. This does not exclude that the total number of visits did not differ. However this was impossible to estimate with a movie played on real time, because the bees were too fast and too numerous to follow. Perhaps if specialised software was used to play the movies in slow motion, it might be possible to estimate the real number of visits during the 10 minutes of experiments.

A. mellifera did show a shorter duration of visits to herbivore infested plants compared to control plants. Just as *P. rapae, A. mellifera* did not show a correlation between the duration of visits and the amount of nectar present in the flowers. There probably were other aspects underlying the difference in duration of visits between the two treatments. The quality of nectar could have influenced the duration of visits. The sugar content of nectar could have decreased due to herbivory (Mutikainen & Delph,1996), but it was not measured in this study. Bees are known to be attracted by nectar with a high sugar content and pollen with a high nutritive value (Detzel & Wink, 1993).

In certain plant species honey bees are also attracted by secondary compounds in the nectar, probably because the plant selects his pollinator on pollination effectiveness and flower constancy (Lui *et al*., 2007). These nectars often have high sugar concentrations to mask the unpleasant taste of the secondary compounds (Gegear *et al*., 2007). Glucosinolates were present in nectar of *B. nigra,* but the question arises whether *A. mellifera* is deterred or attracted by them. If it is hypothesized that the concentration of glucosinolates increases due to herbivory, the results suggest that *A. mellifera* is deterred by the glucosinolates, because the duration of visits was shorter on the herbivore infested plants. Detzel and Wink (1993) tested for several secondary compounds whether honey bees were deterred or attracted by them. They also tested glucosinolates, which acted as deterrents for the honey bees. *Apis mellifera* is often attracted to relatively low concentrations of secondary compounds in nectar, and when concentrations are too high they got deterred by them (Hagler & Buchmann, 1993).

The beehive was put in the gauze tent where the experiments were performed, and was left there during the experiments. In a pilot study there has been experimented with letting a few bees get out of the hive and then closing the hive and start the experiments. The bees soon started to panic because they could not go back to the hive, and thereby stopped foraging. This could be expected because the first bees leaving the hive are often the ‘scouts’ which examine the surrounding for potential food sources. If a potential food source is discovered, the scouts go back to the hive to recruit the ‘recruiter’ foragers. If they cannot reach those recruiter foragers it is logical they get stressed out (Visscher & Seeley, 1982). For the pollinator choice experiments this meant that a whole bee colony was used, instead of a few individual like done with *E. balteatus* and *P. rapae.* Another big difference was that the butterflies and syrphid flies were naive, because individuals were used just for one

experiment, while all the bees were used for all the experiments. Previously experienced scents by a colony of *A. mellifera* can influence the flower choice of foraging individuals. These scents may originate from the food stored in the hive, from the dancing bees and food samples passed on by dancing bees to attending bees (Jakobsen *et al*., 1995). For this study this could mean that the first bees foraging in the first experiments, determined which plants were visited in the other experiments. However, there was no difference in preference for one of the treatment in the number of visits.

The use of social insects, like *A. mellifera,* for experiments like these has got some limitations. When foraging is considered, it should be noted that foraging by a bee cannot been seen as foraging for the individual bee itself, but for the colony. By laying odour trails, piloting other foragers and performing dances, a honey bee constantly communicates with the rest of the colony (Visscher & Seeley, 1982). Therefore the behaviour examined in this study, should be considered as the behaviour of a colony not of individual bees.

To design a similar experiment, comparable with the pollination choice experiments of *E. balteatus* and *P. rapae,* solitary bees could be used. Certain species nest in isolation, while others nest gregarious but without a division of labour like social bees have. Female solitary bees collect nectar and pollen to store in the nest for her offspring. There are species known that discriminate between flowers, for their amount and quality of rewards (Wcislo & Cane, 1996). In the study of Strauss *et al* (1996) the solitary bee visits increased in relation to increasing flower number on a plant.

Pollinator effectiveness of *Pieris rapae*

Stebbins (1970) formulated the so called “most effective pollinator principle”, in which he proposes that selection favours the specialisations in a plant that attracts the visitors providing the most visits and the highest pollination effectiveness. This pollination effectiveness of species is influenced by many aspects. The duration of flower visits for example, is positively related to pollinator effectiveness. Morphological aspects as tongue length and body size can also contribute to the differences in effectiveness. Some studies show that insects foraging for pollen are often less efficient than those foraging for nectar (Sahli & Conner, 2007). Differences in physiology, and foraging behaviour of pollinators can also result in differences in pollination effectiveness (Olsen, 1997).

There is little known of the pollination effectiveness of *P. rapae*. Lazr and Barrows (1984) found that *P. rapae* butterflies carried small pollen loads compared to other species, and are therefore acting primarily as nectar thieves, rarely pollinating plants. However, in other experiments, *P. rapae* butterflies were the most frequent pollinators of wild radish in the USA (Stanton *et al*, 1989).

This research showed that *P. rapae* did pollinate the *B. nigra* plants. It could be concluded that *P. rapae* is an effective pollinator of *B. nigra*. *P. rapae* butterflies carry pollen primarily on legs and tongue, and a far smaller pollen load compared to the pollen load of honeybees and syrphid flies (Lazr & Barrows, 1984). Sahli and Conner (2007) also found that large lepidoptera removed very few pollen during a visit, probably because they fed on nectar. However they still caused a high seed set, suggesting they do not need to remove much pollen in order to effectively transfer pollen to a receptive stigma. Lazr and Barrows (1984) also observed that stigma's were often touched by both tongue and legs. In a natural environment *P. rapae* butterflies show similar flower constancy for several plant species like some Hymenopteran species (Lewis, 1989). By visiting one plant species, this butterfly can be an effective pollinator for this plant species, which also contributes to a more effective pollination. Conner *et al* (1995) even found in their experiments that *P. rapae* were more effective in transferring pollen than honeybees.

Although the control plants did not receive any pollinators and *B. nigra* is a self incompatible plant species (Conner & Neumeier, 1995), seed set still occurred. On the other hand, the plants were raised and transported to the greenhouse compartment in a close range of each other, which could also have caused pollination just by flower touching. Nevertheless there was still a significant difference in the number of seeds set by the control en herbivore plant.

That *P. rapae* was an effective pollinator for *B. nigra* in this study, does not necessarily mean that they would be in nature. The density and flower constancy of *P. rapae* butterflies would probably be smaller compared to the ones used in this study, although *P. rapae* were the most frequent pollinators of wild radish in an field experiment in the USA (Stanton *et al*, 1989). To determine the pollination effectiveness of *P. rapae* in the wild, field studies should be performed. Although it would be difficult to measure the pollination effectiveness of one pollinator in the field, because other pollinators will be present and are difficult to exclude. Different methods than seed set could be used to determine the pollination effectiveness. For example the pollen load on a pollinator, the probability of contacting stigma's and anthers by that pollinator or quantifying the effectiveness as the amount of removal and deposition of pollen (Sahli & Conner, 2007).

Conclusions

The pollinators of this study were affected by herbivory in number of visits and duration of visits, although there was no correlation found between the amount of nectar and duration of visits. Something is making the flowers of the herbivore induced plants less attractive than those of the control plants. Glucosinolates were present in the nectar and if they would increase in concentration due to herbivory of *B. nigra,* this could be an explanation.

Secondary compounds are also found in pollen (Detzel and Wink, 1993). *E. balteatus* primarily fed on pollen during the experiment, thus the change in foraging behaviour is probably caused by changes in the pollen. Glucosinolates might also be present in pollen, and thereby influenced the flower choice of *E. balteatus*.

Another reason for the change in foraging behaviour of the pollinators due to herbivory can be a change in volatiles emitted by the plants. Jakobsen *et al* (1995) found that bees associated floral rewards with floral scents. The volatiles emitted by flowers might change due to herbivory. But pollinators might also be influenced by the green leave volatiles which are produced in plants after herbivory to attract natural enemies (Schoonhoven *et al*., 2005).

Further recommendations

In this study the pollinators, *E. balteatus, P. rapae and A. mellifera* showed changes in foraging behaviour in terms of number of visits and duration of visits, but a clear reason that could have caused these differences was not yet found. Changes in floral volatiles might be an explanation. To examine this, similar techniques as used by Honda *et al* (1998) could be used. In that study floral volatiles were analysed by using Gas Chromatography.

In this study the sugar concentration could not be measured simultaneously with the glucosinolates analyses. Thereby, more nectar samples should be collected to examine the sugar concentrations separately from the glucosinolates analyses.

As mentioned before, the amount and quality of pollen could also be examined, because especially female syrphid flies forage on pollen in stead of nectar. Also the abundance of glucosinolates in the pollen could be examined.

The reaction of natural pollinators in the field could also be examined. The same set up as used in the greenhouse compartment could be used, by applying the same herbivore treatment, and following individual plants for a certain time span and observing the number and duration of different pollinators.

Glucosinolates were present in the nectar of *B. nigra*, but the amount of nectar was insufficient to research differences in concentrations between nectar from herbivore infested and control plants. If differences would appear in future research, bioassays could be performed. In these bioassays the preference of different pollinators for different concentrations of glucosinolates in nectar could be examined.

For a broader view of the reactions of pollinators to herbivory more pollinators could be examined. As mentioned earlier solitary bees could be used instead of, or compared with honey bees. Bumblebees could also be used as extra pollinator.

More flowering plant species could be used for the experiments, to examine if they show the same reactions in means of pollinator attraction.

As mentioned earlier, the volatiles emitted by the flowers could be analysed, to see whether they differ due to herbivory. When differences are found, y-tube experiments could be used to examine which compounds are attracting or deterring the different pollinators. Y-tube experiments could also be used to test whether green leave volatiles, emitted by plants after herbivory, affect the flower choice of pollinators.

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6. Appendix

Appendix 6.1 Number of *E. balteatus* visiting leaves

	Control plants	Herbivore infested plants
Number of Episyrphus balteatus visiting leaves	14	24

Appendix 6.2 Number of *P. rapae* visiting leaves

	Control plants	Herbivore infested plants
Number of Pieris rapae visiting leaves	10	22

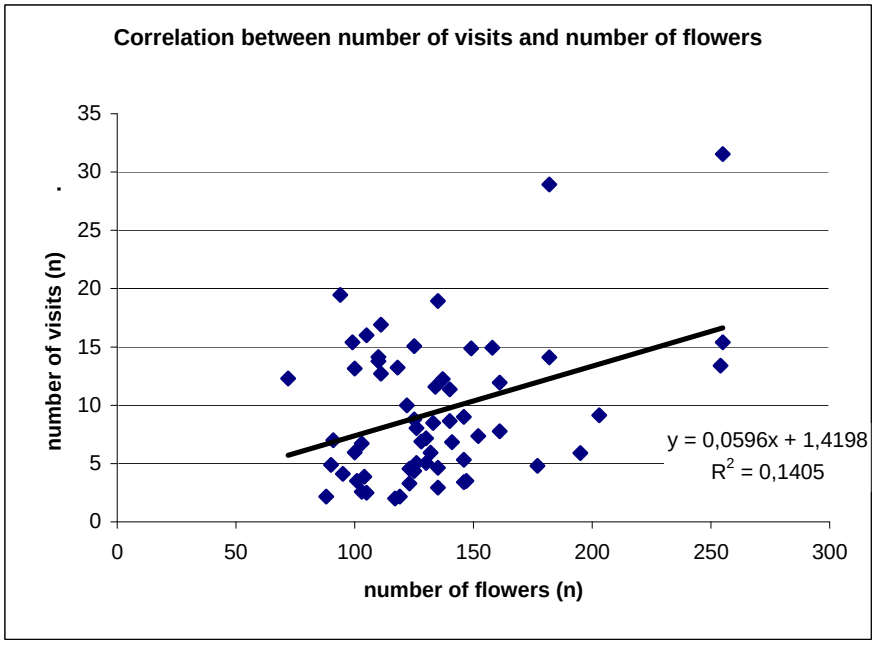
Appendix 6.3 Number of ovipositing females of *P. rapae.*

	Control plants	Herbivore infested plants
number of ovipositing *Pieris rapae*	11	9

Appendix 6.4 Difference between *P.rapae* pollinator choice series with and without trips (Mann Whitney U test)

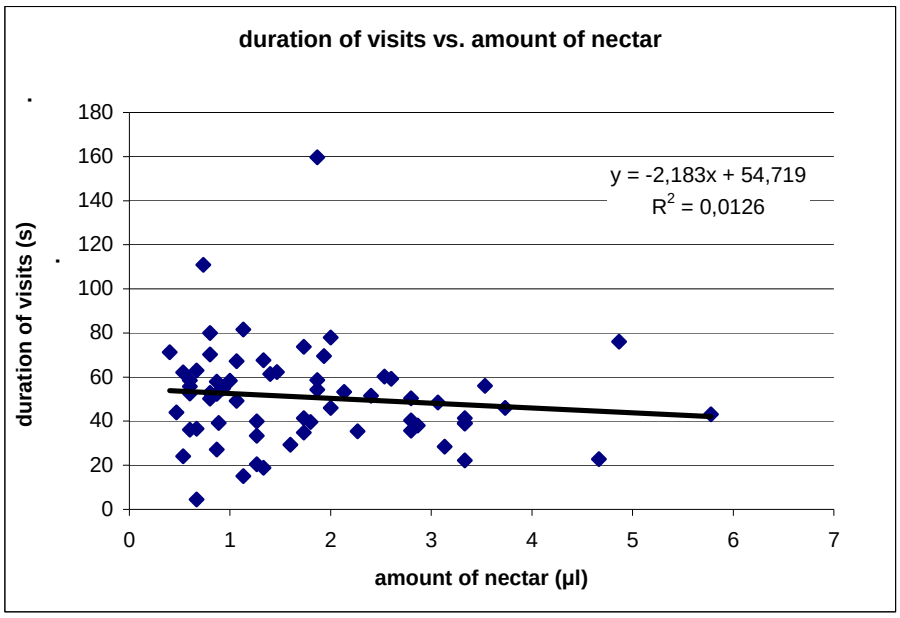
	Herbivore infested vs. herbivore infested with trips	Control vs. control with trips
Number of visits	0.187	0.734
Duration of visits	0.001	0.037

Appendix 6.5 Correlation between number of visits and number of flowers by *A. mellifera*.



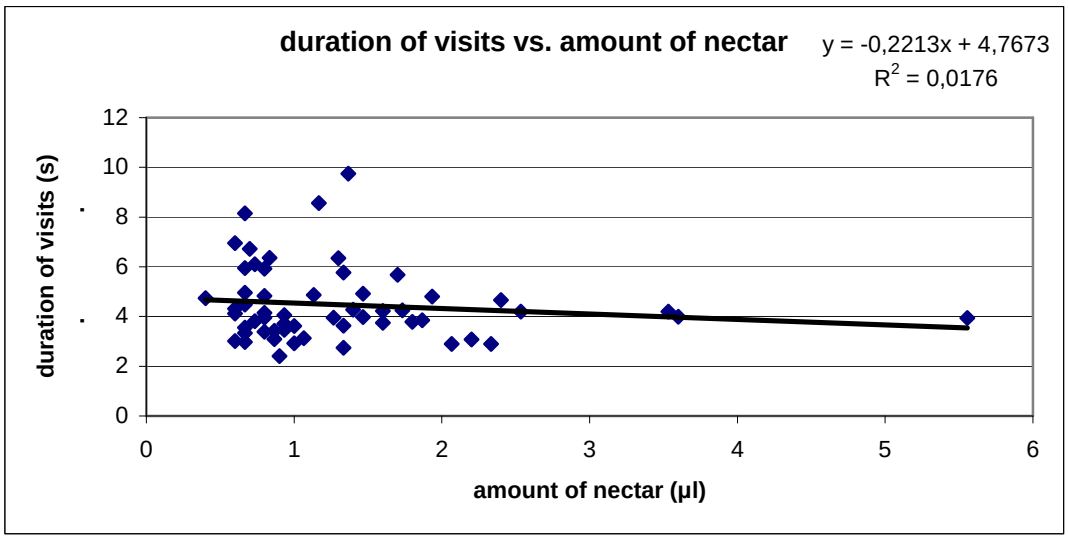
Appendix 6.5: Correlation between the number of *Apis mellifera* visits and the number of flowers of the plants visited (N= 30, *P*= 0.100, Spearman rank test).

Appendix 6.6 Correlation between duration of visits and amount of nectar by *P. rapae*



Appendix 6.6: Correlation between the number of *Pieris rapae* visits and the number of flowers of the plants visited (N= 64, *P*= 0.242, Spearman rank test).

Appendix 6.7 Correlation between the duration of visits and the amount of nectar by *A. mellifera.*



Appendix 6.7: Correlation between the number of *Apis mellifera* visits and the number of flowers of the plants visited (N= 56, *P*= 0.343, Spearman rank test).