

# Gall Midge Olfaction and its Role in Speciation

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Doctoral Thesis  
Swedish University of Agricultural Sciences  
Alnarp 2013

Acta Universitatis agriculturae Sueciae

2013:6

Cover: Male swede midge (*Contarinia nasturtii*) antenna

ISSN 1652-6880

ISBN 978- 91-576-7763-1

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Print: SLU Repro, Alnarp 2013

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### Abstract

With the swede midge (*Contarinia nasturtii*) as our main model species, we study two types of olfactory cues that are of importance for gall midges: 1) the pheromones emitted by the female to attract the male; 2) and the host plant volatiles that the females use when finding a host for oviposition.

We found that both the blend of compounds and the enantiomeric form are important for male attraction in the wind tunnel and in the field. For pheromone reception, the gall midges use the sensillum type that display sexual dimorphism, male swede midge use the gall midge specific sensilla circumfila while the Hessian fly (*Mayetiola destructor*) use s. trichodea.

In a detailed study of the female host finding behavior, we found that olfactory cues are important for the swede midge host selection. However, this "first impression" can be modulated by later plant characters, such as the physical defense of the host plant, or which host plants are available. By using the electrophysiological technique GC-EAD, we compared the response of 12 gall midge species, including the swede midge, to a blend of 45 plant volatiles to explore the relative impact of host plant chemistry, life-history strategies and the midge phylogeny on the gall midge host plant recognition system.

*Keywords:* Gall midges (Cecidomyiidae), olfaction, pheromone, kairomones, sensilla circumfila, host choice, plasticity, speciation, phylogeny.

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*Likheten mellan lyckan och en amöba är att båda förökas genom delning*

Anonym

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## List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Tina Boddum, Niels Skals, Marit Wirén, Robert Baur, Stefan Rauscher and Ylva Hillbur (2009). Optimization of the pheromone blend of the swede midge, *Contarinia nasturtii*, for monitoring. *Pest Manag Sci.*65, 851–856
- II Tina Boddum, Niles Skals, Sharon R. Hill, Bill S. Hansson and Ylva Hillbur (2010). Gall midge olfaction: Pheromone sensitive olfactory neurons in *Contarinia nasturtii* and *Mayetiola destructor*. *Journal of Insect Physiology* 56, 1306-1314.
- III Tina Boddum, Göran Birgersson, Sharon R. Hill, Bill S. Hansson and Ylva Hillbur (2012). Specificity and plasticity of the swede midge (*Contarinia nasturtii*) host choice (manuscript).
- IV Tina Boddum, Béla Molnár, Göran Birgersson, Sharon R. Hill, Bill S. Hansson, Kibrom Abreha, Erik Andreasson and Ylva Hillbur (2012). Swede midge attraction to *Arabidopsis* with altered odor profiles (manuscript).
- V Béla Molnár, Tina Boddum, Göran Birgersson, Sharon R. Hill, Bill S. Hansson and Ylva Hillbur (2012). Ecology and phylogenetic relationship shapes the olfactory systems of the gall midges (manuscript).

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The contribution of Tina Boddum to the papers included in this thesis was as follows:

- I Field experiments, data analysis for both laboratory and field experiments, and writing with support from co-authors.
- II Experiments, data analysis, and writing with support from co-authors.
- III Developed the initial idea together with co-authors, conducted the experiments, data analysis, and writing with support from co-authors.
- IV Developed the initial idea together with co-authors, behavioural experiments, data analysis, and writing with support from co-authors. First authorship is shared with Béla Molnár.
- V Developed the initial idea together with co-authors, performed the molecular biology experiments, data analysis, and writing with support from co-authors. First authorship is shared with Béla Molnár.



## Abbreviations

GC	gas chromatography
GC-EAD	combined gas chromatography and electroantennographic detection
GC-MS	combined gas chromatography and mass spectrometry
GLV	green leaf volatile (i.e. hexanol derivatives)
GS	glucosinolates
IR	ionotropic receptor
OR	odorant receptor
ORCO	olfactory receptor co-receptor
ORN	olfactory receptor neuron
SEM	scanning electron microscopy
SSR	single sensillum recording
TEM	transmission electron microscopy



# 1 Project background and goals

This PhD project is part of the Linnaeus project: Insect Chemical Ecology, Ethology and Evolution (ICE3). The main objective of the Linnaeus project is to explore how insects adapt their olfactory mediated behavior to a changing environment by phenotypic and genotypic modulation, from individual adaptation to evolutionary changes. This encompasses processes acting on widely different timescales, from millisecond decisions to evolutionary adaptations over millions of years – in short, plasticity and evolution.

In this thesis I use the gall midges (Cecidomyiidae) as models to study the role of olfactory in speciation. With their specific lifestyle and fast speciation they are good models for this type of study. I study two types of olfactory cues of importance for gall midges, the pheromones and host plant volatiles.

## Project goals

The goal of my PhD project is to explore how the gall midge peripheral olfactory system adapts to the environment. The male response, both behaviorally and physiologically, to the female produced sex pheromone is investigated for a detailed understanding of the signal attracting the male and the mechanisms for its reception. I also explore aspects of specificity and plasticity of the female host plant choice. The phylogenetic relationship of the gall midges is re-investigated using molecular methods and compared with their response to plant volatiles. By comparing the response of closely and distantly related gall midges the following questions are addressed: Do distantly related gall midges associated with the same host plant use the same or a similar set of odors to identify it? And/or, do closely related species that have different host plant requirements respond to odors common for the different plants?

## 2 Insects as models in evolution

Insects were among the first animals on land, and the diversity and distribution of now living insects is astonishing. With one million species, insects are the most diverse organisms in the history of life – both in numbers of species and variety of structures and behaviors (Grimaldi & Engel, 2005). Several hypotheses that explain the diversity of herbivorous insects have been proposed. One theory is that herbivorous insects and their host plants are involved in "an arms race" through reciprocal evolution/co-evolution (Ehrlich & Raven, 1964). It is, however, debated if the plants are affected by the herbivorous insects or if many insects just follow the evolution of the plants (Jermy, 1984).

Depending on the breadth in their host plant use, insects are classified as specialist or generalists (Schoonhoven *et al.*, 2005). Host plant specialists do, in nature, only feed on one or a few closely related plant species (also called monophagous) or on several species belonging to the same plant family (oligophagous) whereas host plant generalists feed on many plants belonging to different families (polyphagous). While generalists have the advantage of utilizing a large range of resources, they are, compared to specialists, less adapted to a particular resource (Stilmant *et al.*, 2008). Specialists tend to tolerate plant defense better, can manipulate the host to their benefit and have evolved ways to reduce predation and parasitism (Ali & Agrawal, 2012). Evolution of diet breadth in herbivorous insects is suggested to be constrained by limited neural ability (Bernays, 2001). Bernays & Funk (1999) compared a specialist and a generalist population of the aphid *Uroleucon ambrosiae*, and found that both in host finding, host selection, and host acceptance, the host plant specialists made faster decisions than the host plant generalists. Thus, the incorporation of more hosts, and thereby a wider variety of cues to discriminate and choose between, make a host generalists slower at making decisions.

## 3 Insect olfaction and its evolutionary significance

### 3.1 Odorant reception

Insects do locate and select host plant and mate largely based on information from chemosensory cues. The antenna is the main insect olfactory organ, but also the maxillary palps are used in odor detection. The antenna is covered by olfactory sensory hairs, the sensilla. Three types of olfactory sensilla are common in Dipterans: sensilla trichodea, s. basiconica and s. coeloconica (McIver, 1982). The basic morphology of olfactory sensilla is similar (Keil, 1999): their cuticular wall is thin and multiporous, and they are innervated by one to several olfactory receptor neurons (ORNs) surrounded by auxiliary cells. In the sensilla cavity the dendrites of the ORNs are embedded in sensory lymph and connected to the environment through pore tubes (Keil, 1999)

The first step in odor reception is when the odorant molecules enter the sensillum through the pores and the pore tubes (Keil, 1999). Odorant binding proteins facilitate the contact between the odor molecules and the odorant receptors (Ache & Young, 2005). Insects have two types of olfactory receptors: seven transmembrane odorant receptors, ORs and the ionotropic receptors, (IRs) recently identified in *Drosophila* (Benton *et al.*, 2009). Seven transmembrane odorant receptors were originally identified from rodents and are also found in fish and nematodes (Spletter & Lui, 2009), however the insect receptor have inverted topology compared to the others (Benton *et al.*, 2006). The ORs are highly diverse (Leal, 2012), except for the odorant receptor co-receptor (Orco) that shows a high degree of conservation across insect species (Vosshall & Hansson, 2011).

Odorants are detected with a relatively small number of odorant receptors and a combination of strategies. Some receptors are highly selective, responding strongly only to one or a few chemicals whereas others are broadly

tuned and activated by several odorants (Bargmann, 2006; Naters & Carlson, 2006). However, only very few generalist ORNs exist, instead, there are different degrees of specialization (Hansson, 2002).

Receptor activation leads to the generation of action potentials that are transmitted by the ORNs axons to the brain (Naters & Carlson, 2006). Individual neurons show differences in the temporal dynamics and mode (excitation versus inhibition) when stimulated with different odorants (de Bruyne *et al.*, 2001). The axon of the olfactory neuron targets the antennal lobe (AL). ORNs expressing the same receptor protein are scattered over the antenna, but converge in the same region of the antenna lobe, and form structural units called glomeruli (Bargmann, 2006). There are two types of neurons in the antennal lobe (Hildebrand, 1997), the local interneurons (LN) confined to the AL and the projection neurons (PN) that form the signal pathways to higher brain centers, i.e. the protocerebrum, the lateral horn and the mushroom body.

## 3.2 Odorants and odors

The properties of odorants can be defined in terms of their physio-chemical characteristics and they can be transmitted and modulated by the nervous system into the perceived odors. Thus odorant refers to the actual molecule whereas odor refers to the interpreted sensation (Hudson, 2000). Odorants can be classified as chemical signals or chemical cues. In chemical signals, evolution acts both on emission and reception whereas evolution only acts on the reception of a chemical cue (Leal, 2012).

### 3.2.1 Pheromones

Pheromones are chemical signals used for communication within the same species. The term *pheromone* was defined by Karlson & Lüscher (1959) as "substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction". Insect sex pheromones are typically produced and released by the female stimulating conspecific males to fly upwind and locate the calling female (Linn & Roelofs, 1995). As closely related species share biosynthetic pathways, the compounds of their sex pheromone are often similar (Löfstedt, 1993; Linn & Roelofs, 1989) but species specificity is achieved by combining the compounds in unique blends with different ratios and combinations.

### 3.2.2 Host plant volatiles

Plant volatiles are the metabolites that plants emit into the environment (Baldwin, 2010). Some plant volatiles are ubiquitous and released from many different plants whereas others are specific to certain plant families. One type of ubiquitous compounds used in plant-insect interaction are the "green leaf volatiles", six-carbon aldehydes and alcohols, synthesized in and released from green leaves (Hatanaka, 1993). Other plant volatiles are specific to certain plant families, e.g. the glucosinolates, aromatic or acyclic structures containing sulphur and nitrogen, that mainly occur in the Brassicaceae family (Halkier & Gershenzon, 2006).

In insect-plant interactions a series of terms are used to describe how the host cues affect the insect (Schoonhoven *et al.*, 2005) e.g. kairomones are plant volatiles that mediate attraction of feeding and ovipositing herbivores whereas allomones repel or deter the herbivores, or attract natural herbivore enemies or pollinators.

## 3.3 Coping with a complex environment

In their natural environment insects are exposed to many different volatiles in different concentrations and combinations. In this cloud of volatiles the insects have to specifically pick out the relevant signals – food, a host plant that supports larval development (Gripenberg *et al.*, 2010; Craig & Ohgushi, 2002; Singer *et al.*, 1988) and a partner for mating – and at the same time ignore redundant and irrelevant information. However, also relevant signals can vary (Webster *et al.*, 2010) and the insect olfactory system must thus be capable of discriminating behaviorally relevant from irrelevant odors (Lei & Vickers, 2008) but must also be able to respond to some degree of variation. While some behavioral responses are fixed and predictable, others can be modulated e.g. by experience or physiological stage. Saveer *et al.* (2012) demonstrated that the Egyptian cotton leafworm (*Spodoptera littoralis*) switches its olfactory response from food to egg-laying cues after mating. Unmated females were strongly attracted to lilac flowers, a food cue, while after mating they were attracted to cues signaling the larval host plant, cotton.

### 3.3.1 Mixtures – more than the individual compounds

In nature, most olfactory stimuli are blends of single components at certain concentrations and relative proportions. The detection, encoding and discrimination of these blends are an important function of the insect olfactory system (Lei & Vickers, 2008).

The sex pheromones of most insects consist of several simple molecules that together make up a unique multi-component blend (e.g. Groot *et al.*, 2008; Hillbur *et al.*, 2005; Löfstedt *et al.*, 1991). Trimble & Marshall (2008) showed that the complete four compound blend of the obliquebanded leaf roller (*Choristoneura rosaceana*) sex pheromone attract up to 55 times as many conspecific moths in the field as the major compound alone. Furthermore, a wind tunnel study demonstrated that the male response was increase at each stage of upwind flight towards the pheromone source (Trimble & Marshall, 2008). These studies contrast earlier findings, where the compounds found in largest amount, i.e. the major compounds, were assumed to mediate long range attraction while the compounds found in lower amount, i.e. the minor compounds, were suppose to elicited behaviors during close range approach and courtship at the source of the pheromone (Linn *et al.*, 1986).

Compared to the pheromone system – which commonly includes only a few compounds – the number of volatiles emitted from plants and fruits are much higher. Still, insects only use a few key components in a specific combination to detect and locate their host (Webster, 2012; Bruce & Pickett, 2011). For host plant localization, most insects use specific ratios of ubiquitous volatiles (Tasin *et al.*, 2011; Birkett *et al.*, 2004; Honda *et al.*, 1998), which means that host plant recognition does not only require detection of individual compounds but also central processing of the individual compounds (Saveer *et al.*, 2012; Bruce & Pickett, 2011). In addition, some insects use only a few specific plant compounds for their host recognition (Bruce *et al.* 2005) e.g. specialists on plants belonging to the Brassicaceae family, where the specific glucosinolates and their breakdown products, isothiocyanates, i.e. mustard oil flavors are utilized (Hopkins *et al.*, 2009; Städler & Reifenrath, 2009; Barker *et al.*, 2006; Städler *et al.*, 2002; Lamb, 1989).

Under natural conditions, insects do not encounter the odorants from different sources separately. Instead, they are embedded in a background of other odorants. When insects detect their conspecific sex pheromone it is always surrounded by other odorants, mainly plant volatiles (Reddy & Guerrero, 2004). Plant volatiles can synergistically enhance the response of an insect to the sex pheromone (Varela *et al.*, 2011), so that the response to the joint blend is greater than that to the combined responses to the two individual components. The detection of sex pheromone and host plant odorants occur via separated olfactory pathways (Christensen & Hildebrand, 2002), even though interference between them have been found (Pregitzer *et al.*, 2012; Trona *et al.*, 2010).



### 3.3.2 What matters: right or wrong?

In both host and mate finding, insects have evolved mechanisms that maximize the use of the available information in the environment. They do not only detect and respond to "positive" volatiles emitted by suitable hosts or individuals of the same species, but also to the "negative" volatiles. For closely related, sympatric species the pheromone components of one species can act as an interspecific inhibitor in the other species (Birgersson *et al.*, 2012; Löfstedt *et al.*, 1991). Furthermore, insect are not only attracted to the emitted host plant volatiles, but also repelled by volatiles emitted from non-hosts (Linn *et al.*, 2005; Zhang & Schlyter, 2004). The repellent effect can be caused by compounds not found in the host as well as by compounds emitted by the host in doses higher than natural, or when not perceived with the other compounds in the host blend (Webster, 2008).

### 3.3.3 Host range plasticity

To maximize its fitness in a variable environment, an organism can express different phenotypes (Agrawal, 2001). The evolution of adaptive phenotypic plasticity has led to the success of organisms in new habitats and might play a role in genetic differentiation and speciation. In many insects, the female prefers the host plant that best supports larval development (Gripenberg *et al.*, 2010; Craig & Ohgushi, 2002; Singer *et al.*, 1988). However, there are examples of females choosing a poor host (Gripenberg *et al.*, 2007; Harris *et al.*, 2001; Thompson, 1988; Wiklund, 1975) and of females making oviposition mistakes, e.g. due to the lack of the preferred host (Larsson & Ekblom, 1995). If the alternative hosts support larval development, a female oviposition mistake might be the first step in speciation. Nylin & Janz (2009) argue that if inclusion of a new host is important for fitness, there will be selection for co-adaptation of traits involved in metabolism and host finding and genetic accommodation will thus follow a host shift. As a consequence, colonization of a closely related host species or re-colonization of an ancestral host plant will be easier as the existing genetic machinery for host plant recognition and plant metabolism may already fit to some degree (Nylin & Janz, 2009).

Phenotypic plasticity may have facilitated the host shift of *Rhagoletis* flies from hawthorn to apples. *Rhagoletis* use volatile compounds emitted from the surface of fruit to recognize their host plants (Nojima *et al.*, 2003a; Nojima *et al.*, 2003b; Zhang *et al.*, 1999) and individuals derived from a given host have a strong preference for that compared to non-natal host (Linn *et al.*, 2005; Linn *et al.*, 2003). *Rhagoletis* flies mate on or near the fruit of their host plant (Linn *et al.*, 2003; Feder *et al.*, 1999). Consequently, differences in host preferences translate into mate choice. Therefore, the phenotypic plasticity in the host

choice by *Rhagoletis* flies allows the colonization of a new habitat. However, there are other factors, such as allopatry and temporal isolation, that also restrict the gene flow between organisms in the new and the original habitat (Agrawal, 2001).

### 3.4 Olfaction and speciation

From an evolutionary perspective there is a strong selection pressure for a specific and sensitive olfactory system. Behaviors essential for the fitness of an individual, such as mating and habitat choice, are to a large extent driven by olfaction. Many male insects use the female-produced sex pheromone in mate seeking while the females themselves use plant-produced volatiles to find an appropriate host for its offspring.

As described above, speciation in plant-feeding insects can be associated with a host-plant shift. A genetically based oviposition preference of adult females, different performance of the offspring on different host plants or a combination of both, reduce the probability of encounters between individuals associated with different host plants (Groot *et al.*, 2010). Divergent selection and adaptation to the conditions associated with the new plant will further drive speciation (Funk *et al.*, 2002; Berlocher, 2000; Groman & Pellmyr, 2000).

A common type of behaviorally reproductive isolation in moths is associated with sexual communication (e.g. Thomas *et al.*, 2003). There is strong stabilizing selection for fine tuning between the pheromone "sender" and the "responder" as the pheromone (and the capacity to respond to it) is directly associated with reproductive success (Löfstedt *et al.*, 1991). Females emitting the species specific pheromone blend will be attractive to the majority of males and the males responding to the most common pheromone blend have the possibility to mate with most females. If there is risk for hybridization, additional separation can evolve e.g. the pheromone component from one species act as behavioral antagonists to other species (Linn & Roelofs, 1989; Löfstedt & Vanderpers, 1985). However, the numerous insect pheromones demonstrate that despite strong stabilizing selection, sexual communication systems still evolve (Hall *et al.*, 2012; McElfresh & Millar, 1999; Löfstedt *et al.*, 1991). Groot *et al.*, (2006) showed, that communication interference between closely related sympatric species, exert directional selection that counteracts the intraspecific stabilizing selection. This counteraction can explain how pheromone systems can change and how diverse pheromone systems evolve.

## 4 The gall midges

Gall midges (Cecidomyiidae) are excellent models for studying the role of olfaction in speciation, as olfactory-based decisions are directly linked to the fitness of the midges. The females use volatiles emitted by the host for localization of oviposition site, and the males use the female produced sex pheromone for mate localization.

The gall midges are a fast diverging Dipteran family containing more than 5000 described species (Gagné, 2004). The life span of adult midges can be as short as 1-2 h, but is commonly 1-2 days (Harris & Foster, 1999). Within this limited time, the midges have to locate a conspecific partner for mating and the females have to locate a suitable oviposition place (Harris & Foster, 1999). In most species, mating occurs at the site of emergence, the female emits a species specific sex pheromone that attracts the male (Hall *et al.*, 2012). Depending on the life history of the gall midges, different selection pressures act on the olfactory system. Midges associated with annual hosts have to migrate to find the host every season, thus employing long range olfactory cues in the searching, location and identification of a suitable host, while midges associated with perennial hosts emerge in its vicinity and may therefore rely less on olfactory cues.

### 4.1 Host specificity

Gall inducing insects are considered to be among the most host specific insects that exist (Carneiro *et al.*, 2009). At the genus level, some gall midges are host plant generalists – especially in large genera such as *Asphondylia* and *Contarinia*. However, at species level most gall midges are highly specialized to their host (Yukawa *et al.*, 2005) and even to the level of host plant part (Stireman *et al.*, 2008; Joy & Crespi, 2007). Two types of host-associated adaptations are suggested to accelerate gall midge speciation: host shift

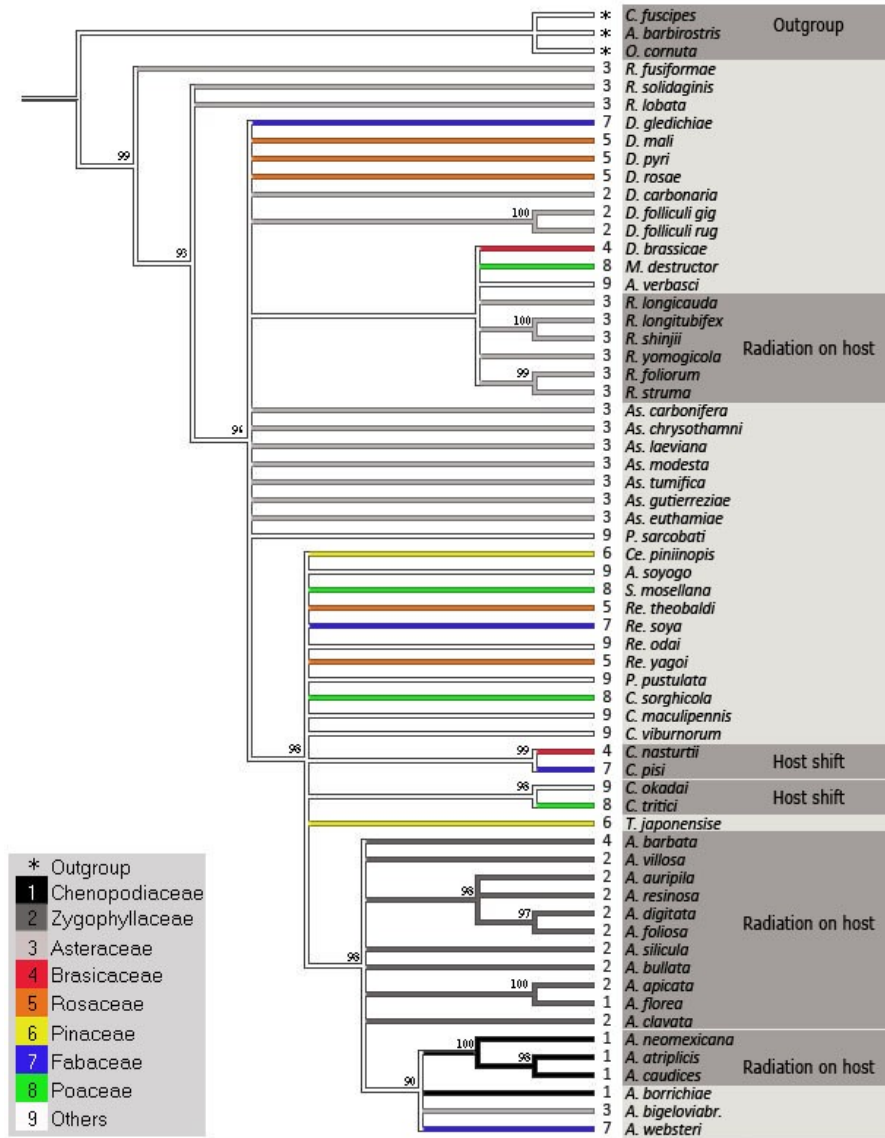


Figure 1. Phylogenetic tree including 60 gall midge species with part of CO1 sequenced. Sequences were obtained from GenBank, and the tree constructed in MrBayes (Huelsenbeck and F. 2001) and visualized using Mesquite (Maddison and Maddison 2010). On the tree, the host plant family of the individual species is indicated, both by color code and numbers. Two types of speciation can be observed in the tree, host shift-induced speciation (e.g. *Contarinia nasturtii* and *C. tritici*) and radiation on one host family (e.g. the *Asphondylia* complex – starting with *A. barbata*).

induced speciation, the result of a shift between two unrelated host plant species (Abrahamson *et al.*, 1994) and radiation, the rapid speciation on one host (Price, 2005). Figure 1 shows the phylogeny and host plant families of 60 gall midge species. Examples of both host shift-induced speciation and radiation on one host family are indicated (Figure 1). The closely related swede midge (*Contarinia nasturtii*) and lemon wheat midge (*C. tritici*) utilize host plants from different families, indicating that a host shift was involved in their speciation. In contrast, all the species in the *Asphondylia* complex are associated with the same host family – a clear example of speciation by radiation.

## 4.2 Sex pheromones

The female sex pheromones have been identified for 16 gall midge species and the similarity among the identified pheromones is striking (Hall *et al.*, 2012). All identified pheromones are straight, odd numbered carbon chains (7-17 carbon atoms) with an acetoxy-, butyryloxy- or keto-functionality group on the

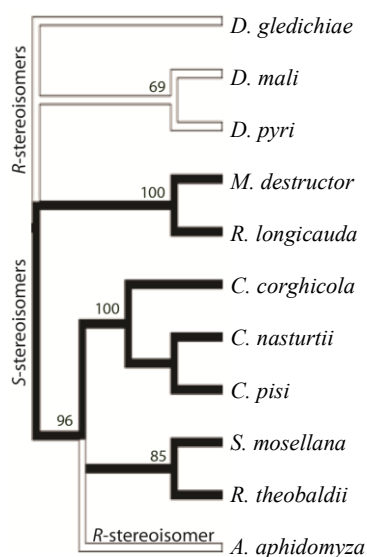


Figure 2. Phylogenetic tree containing the gall midges for which the pheromones are identified and CO1 sequenced. Gall midges that produce pheromones with *R*-stereoisomers are marked in white while species with *S*-stereoisomers are marked in black. Character history was traced using Mesquite (Maddison & Maddison, 2010) under parsimony.

second carbon. The molecules can be saturated or unsaturated and all gall midge sex pheromones identified so far have at least one chiral center. For some gall midge species one or several of the stereoisomers that are not produced by the female inhibit the male attraction. In other species the males do not detect or are not behaviorally affected by the presence of the non-natural stereoisomers. Despite their similarities, the female sex pheromones are species-specific, only attracting conspecific males. The gall midge pheromones have not been studied in a phylogenetic context, thus the evolutionary significance of the inhibitory compounds is not known. However, a comparison of the molecular structure of the pheromone compounds shows that the *S*-stereoisomer is most common, but there are several examples of midges having *R*-stereoisomers as part of their sex pheromone (Hall *et al.*, 2012). The

phylogenetic tree of the gall midges with their pheromone identified (Figure 2), both demonstrate examples where groups of closely related species use the same stereoisomeric form but also examples where closely related species have *R*- and *S*- isomers, respectively, as their pheromone components. This indicates that alterations in stereoisomeric form can be a way for gall midges to ensure species-specificity in the pheromone signal. However, in the gall midges no sister species have yet been found to utilize the opposite isomers of the same component as pheromones. Altering the geometry of the pheromone compounds is, however, a common mechanism that enables species-specific communication within a complex of closely related species based on only a few related compounds. The sawflies (Diprionidae) have a pheromone system where the stereospecificity is important (Anderbrant *et al.*, 2010) and E/Z isomers in Lepidoptera pheromones provide reproductive isolation and prevent mating between sympatric species (Löfstedt *et al.*, 1991).

## 5 How do we know what the gall midges smell?

In the field of insect chemical ecology, numerous techniques are used to unravel what compounds the insect can smell, which are behaviorally active and what they mean to the insects in their natural environment (Takken & Dicke, 2006). In this PhD project I used a multidisciplinary approach to explore gall midge olfaction, ranging from molecular studies of the phylogenetic association of the midges, to behavioral studies both under controlled laboratory condition and under natural conditions of the midges. In addition, morphological and electrophysiological studies were conducted to investigate gall midge odorant reception mechanisms.

### 5.1 Behavior

To know what odorants are active the behavioral response of the swede midge (*Contarinia nasturtii*) was studied. Wind tunnel and field experiments were performed to compare male attraction to the different pheromone blends. In the wind tunnel individual males were released downwind from the odor source, the test blends applied to a filter paper, and a response was classified as landing or no landing. In the field trials, the attractiveness of Delta traps (PheroNet AB, Sweden) with dispensers containing the different pheromone blends were compared (Figure 3A).

Female swede midge oviposition choice and larval survival were tested by enclosing mated females in cage with either one type of plant or with two different plant types (Figure 3B). The females were given 24 h to oviposit, and the presence and absence of eggs and larvae on the plants were counted after 3 and 16 days, respectively. In a separate cage experiment, the oviposition choice of the individual female was explored. To test whether females oviposit on a

single plant or spread their eggs among several plants one female was enclosed in a cage with six plants of the same type.

Swede midge olfactory-based attraction to different plants was tested in a glass Y-tube olfactometer (Figure 3C). In each replication, 10 to 20 one day old mated females were released simultaneously in the entry arm and the midges were allowed to choose between the two stimuli at the end of each arm. When the females had made a choice, they were trapped in bulbs connected to each side arm (Figure 1C, top right).

## 5.2 Electron microscopy

To visualize the antennal structures electron-and light microscopy was used. A light microscope and scanning electron microscopy (SEM) were used when the outer structure was studied (Figure 7) while transmission electron microscopy (TEM) was used to study the sensilla innervations and the wall structures (Figure 8).

## 5.3 Electrophysiology

Electrophysiological recordings were conducted to reveal what compounds the midges are able to detect.

Combined gas chromatography and electroantennographic detection (GC-EAD) was used to test the response of the entire antenna to a blend of plant compounds. The head of the gall midges was excised and inserted into glass capillaries filled with saline and attached to a reference silver/silver chloride electrode (Figure 3D). The odor sample was injected on the gas chromatograph and the antennal signal was recorded simultaneously with the response from the flame ionization detector (FID) of the gas chromatograph.

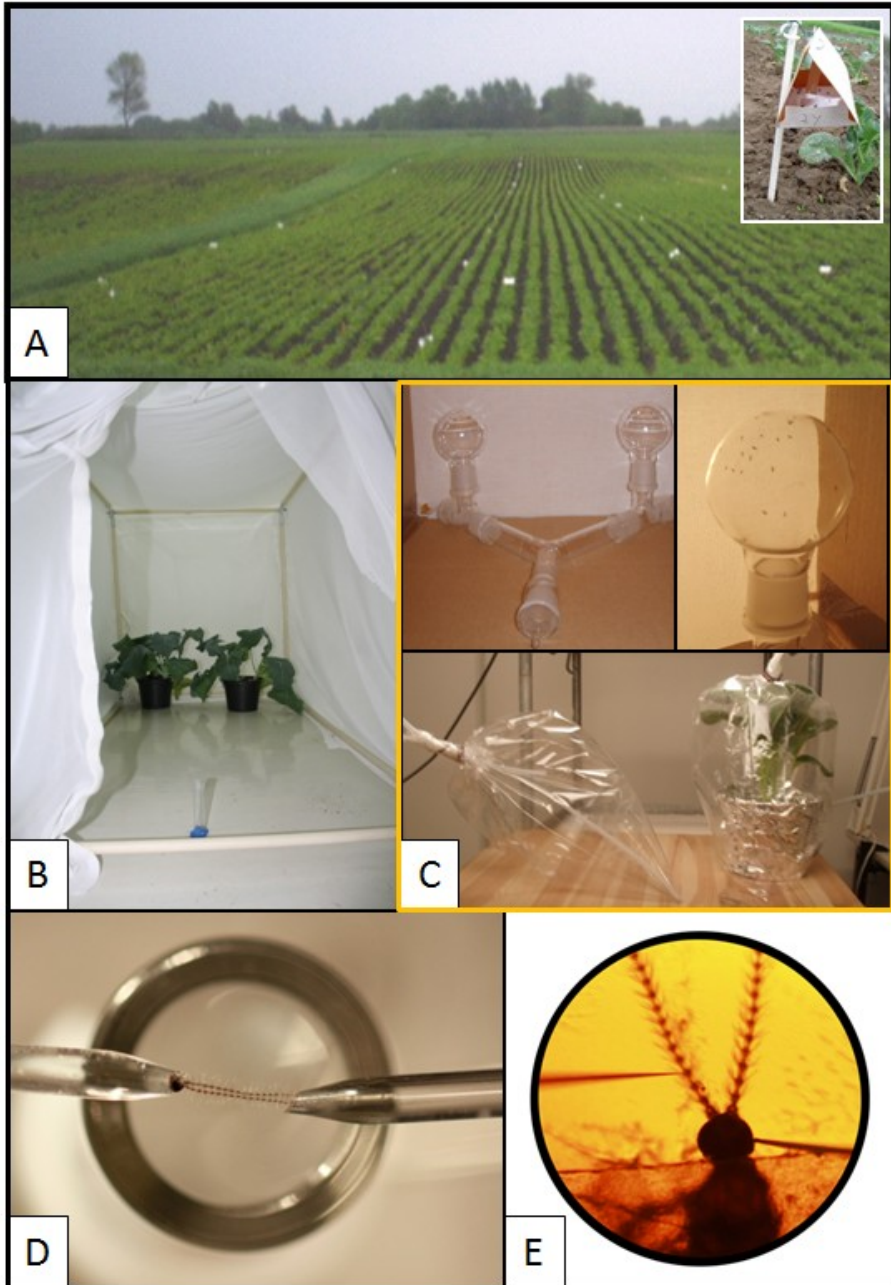
Single sensillum recording, SSR, is an extracellular technique that monitors the electrical events in the medium surrounding the receptor neurons. It was conducted on sensilla circumfila on male swede midge (*C. nasturtii*) antennae and on s. trichodea on male Hessian fly (*Mayetiola destructor*) antennae (Figure 3E). For data acquisition, an electrolytically sharpened tungsten electrode was inserted at the base of a sensillum. The neural activity in response to pheromone components was recorded.

## 5.4 To study the evolutionary changes in olfaction

To get an idea of how the peripheral olfactory system and the host preference have evolved the phylogenetic relationship of the midges was studied. DNA



was extracted and regions of three genes with different molecular clocks were sequenced: the mitochondrial cytochrome c oxidase subunit I (CO1), elongation factor 1- $\alpha$  (ef1 $\alpha$ ) and the 12s ribosomal gene (12s). By combining the three genes, it is possible to obtain a phylogenetic tree that includes both closely and distantly related species. For sequence alignment and the reconstruction of the phylogenetic relationship among gall midges the following programs were used: JModeltest (Posada & Crandall, 1998), SeaView 4 (Gouy *et al.*, 2010), Muscle (build into SeaView 4) and MrBayes (Huelsenbeck & Ronquist, 2001).



*Figure 3.* The techniques used to study gall midge olfaction and host choice: A) field experiments with Delta traps, B) cage for oviposition choice, C) Y-tube, D) gall midge mounted for GC-EAD, E) gall midge mounted for SSR with recording and ground electrodes inserted.

## 6 Summary of results

### 6.1 Recognizing the right female: The emitted signal (Paper I)

In a detailed behavioral study, we examined the robustness of male swede midge (*Contarinia nasturtii*) attraction to the female sex pheromone under laboratory and field conditions. The female-produced sex pheromone is a blend of (2*S*,9*S*)-diacetoxyundecane, (2*S*,10*S*)-diacetoxyundecane and (2*S*)-acetoxyundecane emitted in a 1:2:0.02 ratio (Figure 4). Hillbur *et al.* (2005) showed that the ratio of the three compounds, especially (2*S*)-acetoxyundecane, is important for male attraction in the wind tunnel. When the relative amount of (2*S*)-acetoxyundecane was too high, male attraction was inhibited, whereas male attraction was reduced but not completely inhibited at

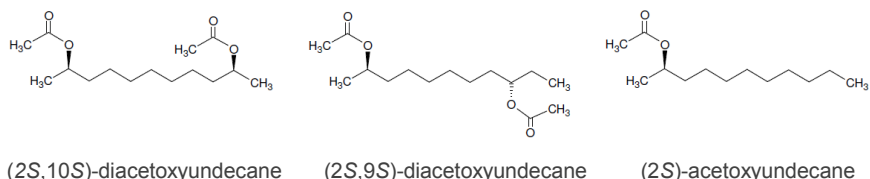


Figure 4. Structures of the swede midge pheromone components

a lower ratio. However, at a low dose of the pheromone blend, males reacted to a blend without (2*S*)-acetoxyundecane equally well as to the blend with all the compounds in the correct ratio. We expected the low-dose situation in the wind tunnel to resemble the situation in the field, where the compounds are released over many days and dispersed over a greater area. Therefore we compared the efficiency of traps with and without (2*S*)-acetoxyundecane (Figure 5). Furthermore, (2*S*)-acetoxyundecane passively evaporates faster than the two diacetates, and thus, a positive effect of the monoacetate in the three-

component blend might only be temporary. However, we found that the presence of (2*S*)-acetoxundecane increased the attractiveness of the males of the pheromone blend compared to the two-component blend during the whole test period (Figure 5), except when trap catches were low.

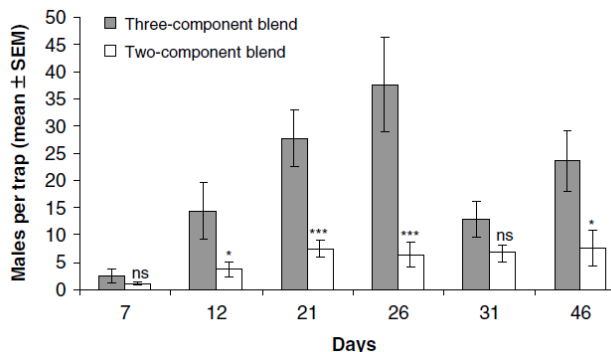


Figure 5. Average number of males caught in traps with either the three-component blend or the two-component blend (n = 10 for each trap type).

The three compounds of the swede midge are all *S*-stereoisomers. However, it was not yet known how the non-female produced enantiomers of the pheromone components affect the males. Our results demonstrated, that both in the wind tunnel (Figure 6A) and in the field (Figure 6B), male attraction was maintained when (2*S*)-acetoxundecane was substituted by racemic 2-acetoxundecane (RAC 2) and when (2*S*,9*S*)-diacetoxundecane was substituted with 2,9-diacetoxundecane (RAC 2,9). However, when (2*S*,10*S*)-diacetoxundecane was substituted with 2,10-diacetoxundecane (RAC 2,10) males rarely showed any activity in the wind tunnel – they rather tried to avoid the plume – and the traps catches did not differ significantly from the blank traps.

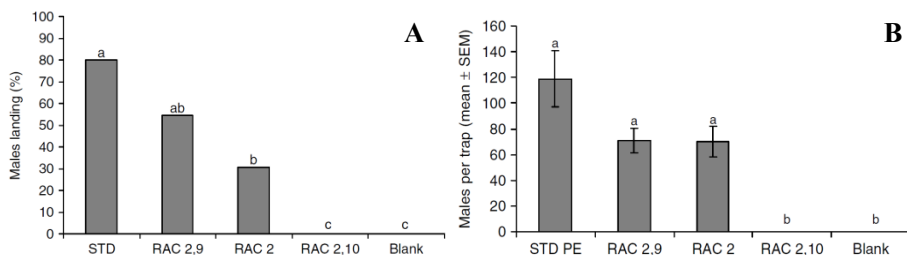


Figure 6. A) Attraction of male swede midge to the different pheromone blends in the wind tunnel and B) average number of males caught in traps in the field. STD: All pheromone compounds in same ratio and stereoisomeric form as emitted by the female. RAC 2,9: All stereoisomers of 2,9-diacetoxundecane, RAC 2: All stereoisomers of 2-acetoxundecane, RAC 2,10: All stereoisomers of 2,10-diacetoxundecane

## 6.2 Recognizing the right female: Pheromone detection (Paper II)

There are two sides of the species-specific signal in gall midges, the female has to emit a unique signal and the male has to be able to receive it. We studied pheromone reception in two species of gall midges, the swede midge (*Contarinia nasturtii*) and the Hessian fly (*Mayetiola destructor*), that both have a multi-component pheromone in which the ratio as well as the isomeric and enantiomeric form of the compounds are of importance for male attraction (Andersson *et al.*, 2009; Hillbur *et al.*, 2005). Gall midges have three types of sensilla that have been suggested to have an olfactory function: sensilla coeloconica, s. trichodea and s. circumfila. Sensilla trichodea and s. coeloconica are common insect sensilla whereas s. circumfila are unique to the gall midges (Harris & Foster, 1999; Gagné, 1989; Slifer & Sekhon, 1971). The sensillum circumfilum is a compound structure evolved from independent sensilla that have merged into one structure encircling the antennal segments (Hallberg & Hansson, 1999). There are two morphological types of s. circumfila. One type is located close to the antennal surface and the other type consists of elongated loops protruding from the antennal surface (Hillbur *et al.*, 2001; Crook & Mordue, 1999; Solinas & Nuzzaci, 1987; Slifer & Sekhon, 1971). Interestingly, males with protruding sensilla circumfila tend to have few s. trichodea, while they are common in males that have s. circumfila located close to the antennal surface. In both the swede midge and the Hessian fly, we found the same sensillum types that had been described in other gall midges, sensilla trichodea, s. coeloconica, s. chaetica and s. circumfila (Figure 7). We focused on the two olfactory sensilla that displayed sexual dimorphism: the male swede midge sensilla circumfila (Figure 7A) that are enlarged compared to the females (Figure 7B) and the Hessian fly s. trichodea that are more numerous in males (Figure 7C) than in the females (Figure 7D). Swede midge s. circumfila consist of approximate 10 sensilla per node fused to a continuous wreath that encircle each node (Figure 7A). Each sensillum is innervated by one sensory neuron (Figure 8A) that branches as the dendrites invade the sensilla branches (Figure 8B, C). A cross section of the area in which the two adjacent sensilla fuse revealed that the cuticle of the two sensilla form a continuous external structure, however, it could not be conclusively determined whether lymph is confluent between sensilla (Figure 8-C2). The morphology of Hessian fly s. trichodea is typical for this sensillum type. It is innervated by two un-branched sensory neurons (Figure 8D, E) and perforated by pores (Figure 8-D2).

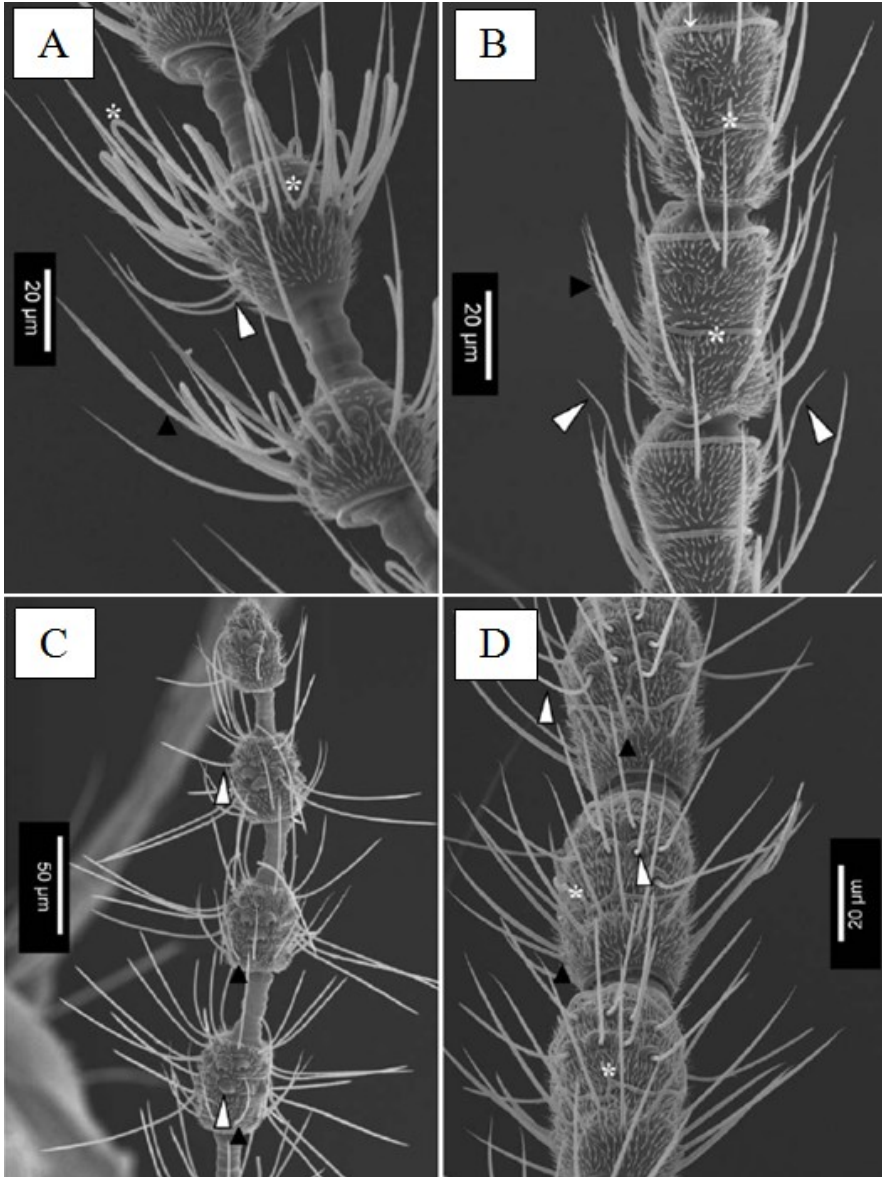
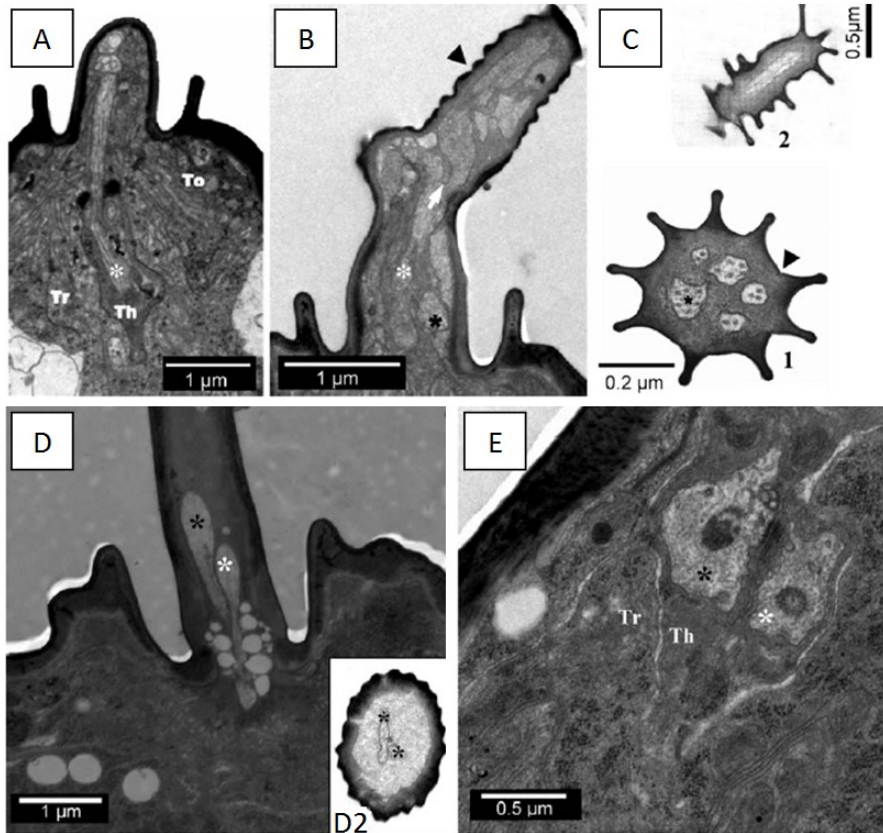


Figure 7. Scanning electron microscopy of swede midge male (A) and female (B) as well as Hessian fly male (C) and female (D) antennal structures. Three sensillum types are visible on the antenna of both species: sensilla circumfila (\*), s. trichodea (Δ) and s. chaetica (▲)



*Figure 8.* Transmission electron microscopy of male swede midge sensilla circumfila, the innervating cell (A and B), the sensilla pores (C1) and the area in which the two adjacent sensilla fused (C2). Olfactory cells in male Hessian fly s. trichodea (D), the sensilla pores (D2) and their support cells (E)

The single sensillum study demonstrated that it is the sexually dimorphic sensilla that respond to the sex pheromone blend, i.e. swede midge sensilla circumfila and Hessian fly s. trichodea (Figure 9). In the swede midge, all responding cells were activated by the pheromone blend, except for two that only responded to the behaviorally inhibitory stereoisomers of 2,10-diacetoxyundecane. In male Hessian fly the single sensillum recordings revealed spikes with two different amplitudes, indicating activity of both cells innervating each sensillum. The number of compounds that elicited a response in the cells varied from one to three, in some cases the two ORNs responded specifically to different compounds.

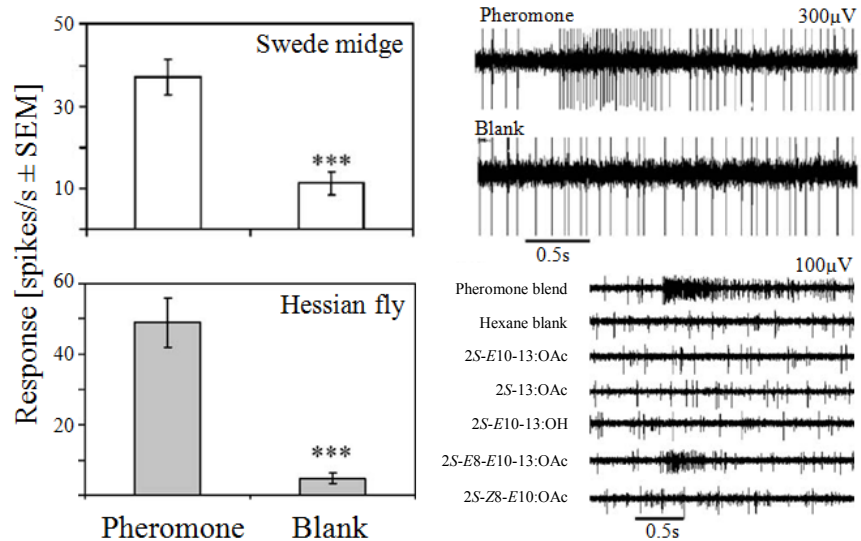


Figure 9. Swede midge (top) and Hessian fly (bottom) response to pheromone compounds.



### 6.3 Specificity and plasticity of host choice (Papers III and IV)

In this study we use the swede midge (*Contarinia nasturtii*), a crucifer specialist, to investigate the mechanisms behind the specificity and plasticity in the female host choice.

We demonstrated that gall midge host specificity is initiated by the olfactory-based host plant choice of the female (Figure 10). In the olfactometer the females were attracted to cauliflower (Figure 10A), ripening rapeseed (Figure 10B) and *Arabidopsis* (Figure 10C), and not attracted to rapeseed at the rosette stage (Figure 10E), wheat (Figure 10G) or lettuce (Figure 10G). The

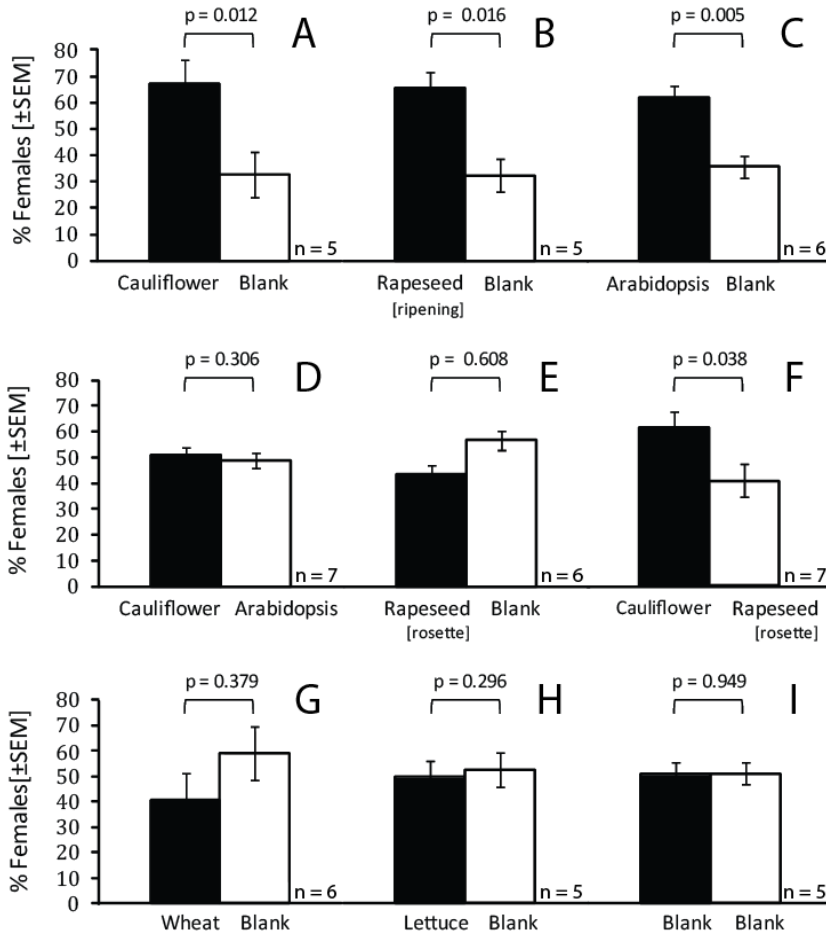


Figure 10. Behavioral response of female swede midge to plants in the olfactometer: (A) cauliflower vs. blank, (B) rapeseed [ripening] vs. blank, (C) *Arabidopsis* vs. blank, (D) cauliflower vs. *Arabidopsis*, (E) rapeseed [rosette] vs. blank, (F) cauliflower vs. rapeseed [rosette], (G) wheat vs. blank, (H) lettuce vs. blank, (I) blank vs. blank.

females were not only able to differentiate between plant species solely based on olfactory cues, but also between different phenological stages of the same plant: the ripening stage of rapeseed was more attractive than blank (Figure 10B) whereas the rosette stage of rapeseed not was significantly different (Figure 10E). To further study what compounds are important for swede midge host attraction, the attraction to *Arabidopsis* with different odor profiles were compared. We found that attraction was maintained both when plants were lacking the ubiquitous green leaf volatiles (GLVs) (Figure 11A) and when the plants were also lacking the crucifer specific glucosionlates (GS) (Figure 11B). The robustness of the female swede midge response to the tested plants, show that insect host localization is complex and may not just be a matter of the right combination of a few compounds, or the presence of certain host unique compounds.

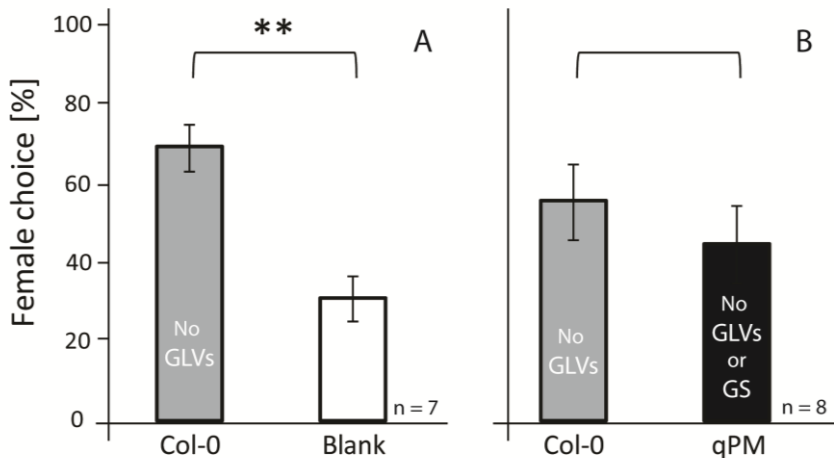


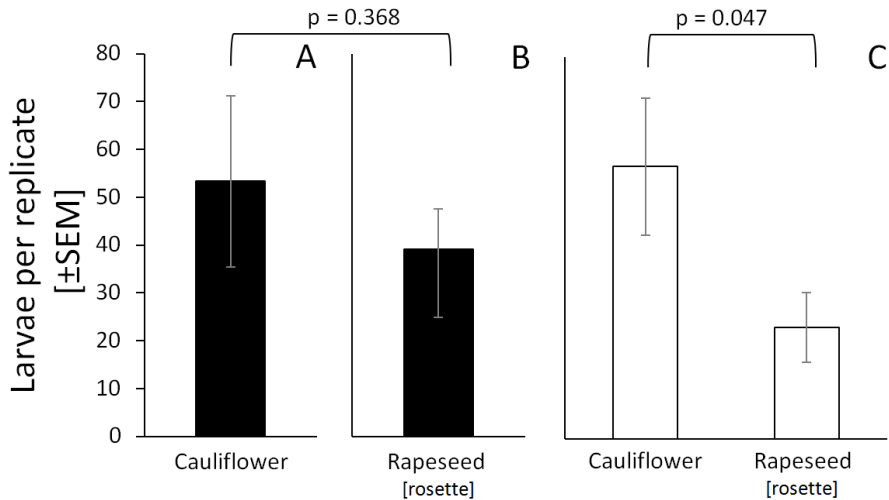
Figure 11. Female swede midge attraction to *Arabidopsis* with no production of green leaf volatiles (Col-0) and to *Arabidopsis* with neither green leaf volatiles nor glucosinolates (qPM).

The olfactory preference of the female, while narrow, encompassed a broader spectrum of host plants than were accepted for egg-laying. No eggs were oviposited on *Arabidopsis*, for which the midge showed a clear olfactory preference. This is likely because of the pronounced physical plant defense provided by "spiky" trichomes covering the leaves and stems (Mauricio & Rausher, 1997). However, when a host plant was accepted by the female for oviposition, the plant supported successful larval development.

Swede midge females displayed plasticity in host plant choice depending on the selection of plants available for oviposition. When females were allowed to oviposit either on rapeseed in the rosette stage (Figure 12A) or on cauliflower (Figure 12B), comparable numbers of larvae could be found on the

plants. However, when the female midge could choose between the two hosts, cauliflower was preferred (Figure 12C).

This slightly broadened range of olfactory preference and plasticity in host choice might be the basis for the rapid speciation reported in the gall midge family. A female that can distinguish a suitable from an unsuitable host, but still accept an alternative host when nothing else is available will have increased fitness compared to a solely discriminative female.



*Figure 12.* The average number of swede midge larvae found on cauliflower and rapeseed [rosette]. (A) Cauliflower in a no-choice situation, (B) rapeseed [rosette] in a no-choice situation, (C) two-choice test with cauliflower and rapeseed [rosette].

## 6.4 Phylogeny, ecology and the peripheral olfactory system (Paper V)

In this study we explore how host plant choice affects peripheral olfactory functions in less related species using the same host plant, and how similar processes occur when closely related species use widely different host plants. We compared the antennal (GC-EAD) response of males and females of twelve gall midge species to a blend of 45 plant compounds with a new molecular based phylogeny of the midges. Figure 13 shows the average response of all males and all females within a species. Only a few compounds elicited responses in all individuals (Figure 13, red squares) or in no individuals (black squares). Instead, there was high within-species variation in the responses, with some, but not all, individuals capable of detecting the compounds (Figure 13, blue squares). The combination of constant and variable responses to different compounds results in a species-specific response profile, defining a physiochemical odor space specific for each species.

The comparison between the neighbor joining trees of the species-specific female (Figure 14, left panel) and male (right panel) response profiles with the molecular-based phylogeny of the gall midges (center panels) demonstrate that neither the female nor the male response trees were completely parallel with the phylogenetic tree (Figure 14). In most gall midge species only the females migrate to the host for oviposition and we thus expected the male and the female response profiles to be different. However, males and females within a species responded to the plant compounds in a similar manner indicating that their olfactory peripheral systems are shaped by the same processes and not by sex-specific processes.

We found that the group of *Dasineura* species responded to the odor panel in a similar manner, except for the crucifer specific *D. brassicae* that responded similarly to the distantly related crucifer specialist *C. nasturtii*. However, the responses of the *Contarinia* species did not reflect their phylogeny. *Contarinia sorghicola* grouped based on its response profiles with the unrelated *M. destructor* that also utilizes a grass host. Interestingly, the *Contarinia* are not limited to a specific host plant genus as many other gall midges (Yukawa *et al.*, 2005), indicating that host plant shift occurs comparatively frequently in this genus – an ecological trait that appears to be mirrored in their olfactory system.



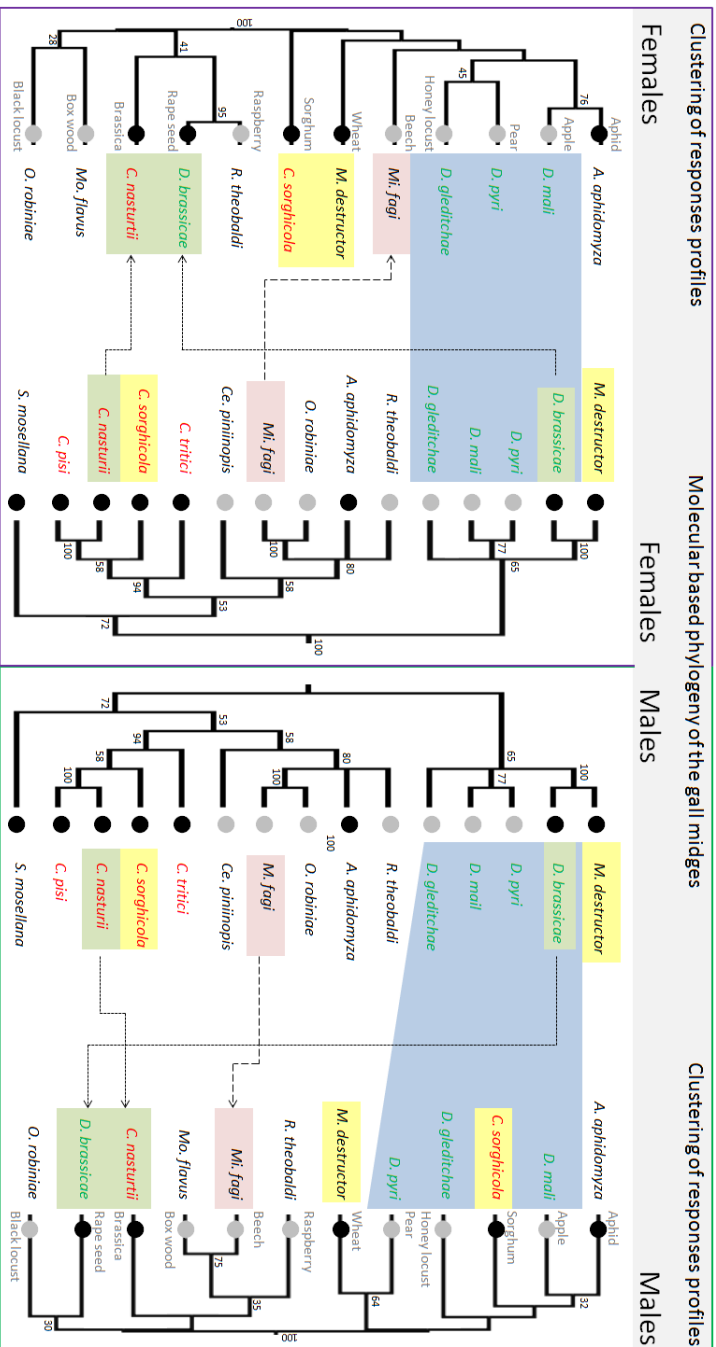


Figure 14. Comparison of the molecular based phylogeny of the midges to the neighbor joining clustering of the responses of the females and males, respectively.

## 7 Conclusion

This thesis describes how the peripheral olfactory system of gall midges adapts to olfactory cues of importance for their fitness. I demonstrated a species-specificity of the swede midge (*Contarinia nasturtii*) sex pheromone, both concerning blend composition and stereoisomeric form of individual pheromone components, that indicate it evolved to prevent mating between closely related sympatric species. Furthermore, I show that male swede midges use the for gall midges unique sensillum type, sensilla circumfila, to detect the female sex pheromone. These findings in combination with our structural data imply that this remarkable structure have evolved to increase spatial and temporal detection of the compounds in a blend.

In contrast to the conservative sex pheromone system, I found plasticity in the female response to host plants. The plasticity was observed both in the female choice of plants for oviposition and in the response to host plant volatiles. The specificity of the gall midges is reflected in the swede midge olfactory-based host plant choice, although, the final oviposition choice is adjusted according to which potential host plants are available.

Combining electrophysiological and molecular studies on twelve gall midge species showed that the olfactory system reflects both midge phylogeny and host preference. Gall midges responded to the blend of plant compounds in a species-specific way, all individuals of a species responded to a few specific compounds. However, for the rest of the compounds there was intraspecies variation, with some but not all individual responding. This variation, in combination with the plasticity in female host choice, can be the basis for rapid adaptation to new hosts and an explanation for the comparatively high rate of speciation in the gall midge family.

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## Acknowledgement

First I would like to acknowledge the members of my supervisor group: *Ylva*, *Göran* and *Sharon*. *Ylva*, when we first met, I did not understand anything you were saying. But with time, you taught me Swedish, gall midge science and about life. *Göran*, you have helped me with the techniques and the chemistry in project, and made some beautiful gall midge pictures. Also, thank you for taking care of the formal stuff at the end of my thesis when it all got a bit complicated. *Sharon*, I believe you know everything! And I am amazed that you don't seem to mind enlighten me. Thanks to *Bill S. Hansson*, for advice and comments on science and writing, and to *Regina Stieber*, (Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, Jena) for teaching me molecular biology.

*Niels Skals*. Tak for at du introducered mig til kemisk økologi, og for at du sendte mig til Alnarp.

*Bela*, my fellow gall midge fan. You are probably the only one in the world who finds the gall midges as exciting and beautiful as I do. I am happy you joined our little gall midge group.

*Sophie*, vi ankom sammen og blev sammen. Jeg tvivler ikke på, at vi har haft kvalitetstid på kontoret. Tak for den hygge og gode mad vi har fået sammen, på det sidste med *Martin* og *Marie*. Du har opnået status som *Maries* bedste veninde.

Tak til min familie, I har udviklet en usædvanlig interesse for galmyg og deres antenner! *Mor & far*, det er dejligt at jeg stadig har mit værelse hjemme og at I holder vores fryser fyldt. *Kim*, du er lige så god til at forklare mig molekylær biologi som til at tømme min opvaskemaskine. *Mormor, Bente & Martin, Annette & co., Jette, Ingo & Kristoffer*: tak for dejlige besøg, weekender og ferier. *Martin* og *Marie*, I har været gode til at holde pumper og samle planter. Udflugter til galmyg inficerede marker har næsten altid været frivillige. Tak for frokoster og aftensmad leveret i Alnarp.

I enjoyed being part of the *Chemical Ecology group in Alnarp*. Thanks for a great scientific and social environment. Thanks to all co-workers and co-PhD students (also all of you who made it before me) for discussions and nice company during every day life, travel and field trips.

Thanks to the following for sending gall midges: M.O. Harris and K. Anderson, Department of Entomology North Dakota State University, USA; P. Riolo, R. Minuz and E. Verdolini, Università Politecnica delle Marche, Ancona, Italy and L. Stringer and K. Colhoun, The New Zealand Institute for Plant & Food Research, Lincoln, New Zealand.

This study was supported by the *Linnaeus initiative* "Insect Chemical Ecology, Ethology and Evolution" IC-E3 (Formas, SLU).