Pheromone production in the butterfly *Pieris napi* L.

Rushana Murtazina

*KTH Chemical Science and Engineering*

*Doctoral Thesis*

*Stockholm 2014*
ABSTRACT

Aphrodisiac and anti-aphrodisiac pheromone production and composition in the green-veined white butterfly Pieris napi L. were investigated.

Aphrodisiac pheromone biosynthesis had different time constraints in butterflies from the diapausing and directly developing generations.

Effects of stable isotope incorporation in adult butterfly pheromone, in the nectar and flower volatiles of host plants from labeled substrates were measured by solid phase microextraction and gas chromatography–mass spectrometry.

A method to fertilize plants with stable isotopes was developed and found to be an effective method to investigate the transfer of pheromone building blocks from flowering plants to butterflies. The anti-aphrodisiac methyl salicylate was not biosynthesized from phenylalanine in flowers of Alliaria petiolata.

Both aphrodisiac and anti-aphrodisiac pheromones in P. napi are produced not only from resources acquired in the larval stage, but also from nutritional resources consumed in the adult stage. Males of P. napi produce the anti-aphrodisiac pheromone from both the essential amino acid L-phenylalanine and from common flower fragrance constituents.

Key Words: Pieris, Lepidoptera, pheromone, aphrodisiac, antiaphrodisiac, biosynthesis, flower volatiles, stable isotopes, incorporation, neral, geranial, methyl salicylate, benzyl cyanide, L-phenylalanine
To my family
Content

1. Introduction 1
   1.1 Aim 1
   1.2 Background 1
   1.3 Mating system 2
   1.4 Butterfly pheromones 4
   1.5 Nutritional resources for butterflies 8
   1.6 Production of aromatic volatiles in flower fragrance 9

2. Materials and methods 12
   2.1 Butterflies 12
   2.2 Flowers 12
   2.3 Collection of head space volatiles, solid phase microextraction (SPME) 12
   2.4 Sampling technique 13
   2.5 Separation and identification 14
   2.6 Ratio of isotope incorporation 15
   2.7 Statistical analysis of data 15

3. Results 15
   3.1 Aphrodisiac production in Pieris napi 15
       3.1.1 Aphrodisiac production in direct and diapause generations 16
       3.1.2 Male mating propensity 16
       3.1.3 The volatiles of the wing scent in direct and diapause generations 17
       3.1.4 Larva and adult $^{13}$C glucose feeding 21
   3.2 Fertilizing plants with L-phenylalanine 21
   3.3 Production of anti-aphrodisiac and related male compounds in adult butterflies 25

4. Conclusions 27

5. Acknowledgements 28

6. References 30

7. Appendix A: Contribution 38

8. Appendix B: Structure of pheromones in Pieris sp. 39
This thesis is based on the following papers and manuscripts, which will be referred to by Roman numerals

I  Seasonal polyphenism in life history traits: time costs of direct development in a butterfly. Helena Larsdotter Mellström, Magne Friberg, Anna-Karin Borg-Karlson, Rushana Murtazina, Mikael Palm, Christer Wiklund

*Behavioral Ecology and Sociobiology 03/2010; 64(9):1377-1383.*

II  Timing of male sex pheromone biosynthesis in a butterfly - different dynamics under direct or diapause development. Helena Larsdotter-Mellström, Rushana Murtazina, Anna-Karin Borg-Karlson, Christer Wiklund

*Journal of Chemical Ecology 05/2012; 38(5):584-91.*

III  Amino acid fertilized flowering plants for studying transfer of pheromone precursors to butterflies. Rushana Murtazina, Ilme Liblikas, Raimondas Mozuraitis, Anna-Karin Borg-Karlson

*Submitted to Oecologia*

IV  Anti-aphrodisiac pheromone production in adult butterflies. Rushana Murtazina, Raimondas Mozuraitis, Yuxin Pei, Christer Wiklund, Anna-Karin Borg-Karlson

*Submitted to PloS ONE*

Paper not included in this thesis

V  Nonvolatile chemical cues affect host-plant ranking by gravid *Polygonia c-album* females. Raimondas Mozūraitis, Rushana Murtazina, Sören Nylin, Anna-Karin Borg-Karlson

*Zeitschrift fur Naturforschung C 01/2012; 67(1-2):93-102.*

Paper I and II are reprinted by the kind permission from the publishers.
Abbreviations and Definitions

ANOVA  Analysis of variance
BALDH  Benzaldehyde dehydrogenase
BEAT   Cetyl-coenzyme A:benzyl alcohol acetyltransferase
BSMT   Benzoic acid/salicylic acid carboxyl methyltransferase
GC-MS  Gas Chromatograph-Mass Spectrometer
PA     Pyrrolizidine alkaloid
PAAS   Phenylacetaldehyde synthase
PAL    Phenylalanine ammonia lyase
PAR    Phenylacetaldehyde reductase
PDMS/DVB Polydimethylsiloxane/divinylbenzene
SAMT   Salicylic acid carboxyl methyltransferase.
SPME   Solid phase microextraction

Bivoltine - two generations per year

Diapause - a period during which growth or development is suspended and physiological activity is diminished, as in certain insects in response to adverse environmental conditions

Photoperiod - the duration of an organism's daily exposure to light, considered especially with regard to the effect of the exposure on growth and development

Polygamy - a mating pattern in which a single individual mates with more than one individual of the opposite sex

Sexual conflict or Sexual antagonism - occurs when the two sexes have conflicting optimal fitness strategies concerning reproduction, particularly over the mode and frequency of mating, potentially leading to an evolutionary arms race between males and females

Fecundity - ability to reproduce

Longevity or life expectancy - the expected life time period
1. Introduction

1.1 Aim

The aim of this research was to investigate sex pheromone production in the adult green-veined white butterfly *Pieris napi* L. (Lepidoptera) and to answer the following questions:

1. Do bivoltine species exhibit similar patterns in pheromone production and concentration in butterflies from the diapausing and directly developing generations?
2. Are males of *P. napi* able to produce aphrodisiac and anti-aphrodisiac pheromones in the adult stage?
3. What are the possible substrates for pheromone production?
4. Are there any simple methods to manipulate flower scent composition of benzenoid rich host plants?

1.2 Background

*P. napi* is a widespread and common species in Europe, Asia and North Africa. Larvae of *P. napi* specialize on a variety of species from the family Brassicaceae including some cultivated plants such as *Brassica oleracea* and *Raphanus sativus*. In central Sweden *P. napi* is bivoltine (it has two generations per year). The adults of the diapausing generation eclose in spring, after spending winter in the pupal stage. Their offspring have a generation time of approximately two months and emerge as adults in mid-summer. The offspring of this summer generation develop until the pupae stage, and hibernate as pupae. The density of the summer generation is usually higher than that of the spring generation (Hearth et al 1984). Butterflies from the diapausing, spring, generation have plenty of time to develop in the pupae stage. In contrast, the directly developing, summer, generation is time stressed because their offspring must have time to reach the pupae stage before the onset of cold weather; hence, development in the pupae stage of this generation is severely affected by time constraints (Abrams et al 1996, Gotthard et al 1999). It is known that photoperiod and temperature determine an individual’s choice of
developmental pathway, whether to enter diapause or direct development, in *P. napi* (Nylin 1994).

### 1.3 Mating system

Butterflies can be polygamous (females and males mate more than once) or monogamous (females and males mate only once). *P. napi* is a polygamous species, with wild females from a central Swedish population mating on average 2.65 times (Bergström et al 2002). A laboratory experiment showed that female mating propensity was related to size with larger females being more prone to remate than smaller females (Bergström et al 2002). Although females benefit from multiple matings (Wiklund et al 1993), with some females mating up to 5 times, 12% of females in the wild mated only once (Bergström et al 2002). The reason behind this variation in female polygamy may be the result of sexual conflict, which is a potentially strong force in male female coevolution (Chapman et al 2003).

When approached by a male, both virgin and mated females exhibit the so-called “mate-refusal posture”, with the female raising her abdomen and spreading her wings. However, virgin females often quickly abandon this posture and fold their wings when accepting to mate with the courting male, whereas mated females display this position for as long as the male continues to court her. It has been suggested that the function of the refusal posture is prolongation of courtship in order to allow the female to assess male potency (Rutowski 1979).

Females of *P. napi* receive direct benefits from multiple mating. Females, mated multiply live longer, maintain egg weight for longer and have higher lifetime fecundity than females mated singly (Wiklund et al 1993). The reason underlying this phenomenon may be that females obtain not only sperm during copulation, but also nutrition as a nuptial gift that the females use to increase their lifetime and fecundity. The nuptial gift can also be regarded as parental investment that increases the number and quality of the males’ own offspring (Kaitala and Wiklund 1995). Radiotracer studies have shown that resources derived from the male’s nuptial gift were incorporated into the eggs (Wiklund et al 1993). Females mated twice lay about 60% more eggs compared to singly mated
females (Wiklund et al 1993, Karlsson 1998). Females that obtained a small spermatophore exhibit more willingness to remate than females which obtained a large spermatophore (Rutowski 1980, Kaitala and Wiklund 1995). There is a strong correlation between male size and the amount of ejaculate that the female receives during copulation. So, large males produce more offspring since they invest more nutrients to female egg production and prolong the duration of female unreceptivity (Kaitala and Wiklund 1995, Bissoondath and Wiklund 1996).

On the other hand the nuptial gift is a costly investment and it takes two or three days to produce a new spermatophore of a similar size to the previous spermatophore (Bissoondath and Wiklund 1996). Dry mass of the second spermatophore decreases compared with the first spermatophore but its water content increases. Virgin males of *P. napi* live longer than mated males. However, there was no direct relationship between the number of copulations and longevity (Ferkau and Fischer 2006).

Female ability to mate is limited for several reasons. The spermatophore obtained by a female *P. napi* at mating can comprise up to 23% of the female’s body mass, which means that the spermatophore must be broken down and digested before the female is able to mate a second time (Forsberg and Wiklund 1989). This ejaculate contains not only sperm and nutrition, but also anti-aphrodisiac pheromones that render females unattractive to courting males, and consequently decreases the likelihood of female remating (Andersson et al 2000). Moreover, mating to receive a second spermatophore is time consuming and interferes with egg-laying (Kaitala and Wiklund 1995). Another reason for female unwillingness to remate can be related to weather conditions; because egg-laying is only possible during favourable weather, and season length decreases northwards, northern populations of *P. napi* are more monogamous than southern populations (Välimäki et al 2008). Moreover, the directly developing generation of *P. napi* has been shown to have a higher level of polyandry than the diapause generation (Larsdotter, Mellström and Wiklund 2010).
1.4 Butterfly pheromone

A pheromone is a single compound, or mixture of several compounds, 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 i.e. definite behaviour or a developmental process (Karlson and Lüscher 1959; Wyatt 2003). Sex pheromones are used to attract mates and provide information about individual quality and gender (Harari and Steinitz 2013). The first aphrodisiac pheromone in butterflies was identified by Meinwald et al (1969) in the Queen Butterfly, Danaus gilippus Cramer (Lepidoptera: Nymphalidae). Two components of a pheromone released from the hairpencil organ were identified as pyrrolizidinone, and trans,trans-3,7-dimethyldeca-2,6-dien-1,10-diol. Males with a lower amount of pyrrolizidinone were not able to seduce females, but an additional amount of synthetic compound restored the ability of males to attract females. Diol functioned as a glue sticking the tiny cuticular "dust" particles with pheromone to the female (Pliske and Eisner 1969). In the closely related species Danaus chrysippus L. (Lepidoptera: Nymphalidae) experiments with radiolabelled compounds demonstrated that pyrrolizidinone is produced from an exogenous alkaloid precursor. In addition, electroantennogram experiments revealed the presence of antennal olfactory receptors for pyrrolizidinone in D. chrysippus (Schneider 1975).

During several decades evidence for the existence of different pheromones in Rhopalocera butterflies was collected. However, only a few pheromones have been identified in this suborder, compared with moths. The reason for this might be that for a long time it was assumed that for daytime-active butterflies visual cues were more important than chemical cues (Scoble 1992). Vane-Wright and Boppre (1993) suggested that visual cues are important for Bicyclus anynana Butler (Lepidoptera: Nymphalidae) male recognition at a long distance, but at a short range pheromone recognition plays a more important role. Hence, the male sex pheromone may play a role in the late stage of courtship. Female pheromones also function primarily at close range, in contrast with moths. Finally, in Bicyclus anynana chemical cues in female mate choice were shown to be equally important as visual cues (Costanzo and Monteiro 2007). Three compounds have been identified as comprising the male sex pheromone (Z)-9-tetradecen-1-ol, hexadecanal and 6,10,14-trimethylpentadecan-2-ol. Interestingly these compounds are
similar to some female moth sex pheromones (Geister et al 2008). What is even more interesting is that females appear able to utilize a pheromone signal to avoid inbreeding; in B. anynana it was shown that inbred males have a reduced production of the male sex pheromone hexadecanal which in turn decreased male mating success (van Bergen et al 2013).

In two closely related species Colias eurytheme Boisduval and C. philodice Godart (Lepidoptera: Pieridae) male wing compounds involved in reproductive isolation have been identified. Male C. philodice produced three n-hexyl esters; myristate, palmitate, and stearate, which were absent in C. eurytheme. A branched hydrocarbon, 13-methylheptacosane, was found on the wings of male C. eurytheme, but not on C. philodice (Grula et al 1980). Preliminary behavioral experiments indicate that the esters, especially n-hexyl myristate, function as species-recognition signals. Two populations of Colias eurytheme and C. philodice demonstrated different component quantities in the male’s wings. Sappington and Taylor (1990) suggested that this variation can be explained not only by inheritance, but also by environmental or developmental sources.

Males of Idea leuconoe Erichson (Lepidoptera: Nymphalidae) emit an aphrodisiac in their hairpencils. Females of I. leuconoe were attracted to an artificial butterfly model with male hairpencil extracts (Nishida et al 1996). Pyrrolizidine alkaloid (PA) derivatives (danaidone, viridifloric β-lactone), aromatics (phenol, p-cresol, benzoic acid), terpenoids (geranyl methyl thioether, (E,E)-farnesol), a series of gamma-lactones (6-hydroxy-4-undecanolides and its homologs), and hydrocarbons ((Z)-9-tricosene, etc.) were the major components of this extract. Application of a mixture of all these components to artificial hairpencils induced acceptance posture in females; however, electroantennogram responses on the female's antennae were induced only by viridifloric β-lactone and danaidone. Interestingly, males of I. leuconoe do not demonstrate pharmacophagous behaviour towards pyrrolizidine alkaloid containing plants. Larvae of this butterfly specialize on Parsonsia, a pyrrolizidine alkaloid containing genus (Edgar and Culvenor 1975, Abe et al 1990, 1991), and exhibit some preference for simple phenolic compounds (Nishida et al 1996).

Males of Heliconius charithonia L. (Lepidoptera: Nymphalidae) are even able to discriminate between male and female pupae. Females produce sex recognition
pheromone linalool oxides (furanoid) at the end of the pupae stage. Since linalool oxides (furanoid) are widespread compounds, the authors suggested that, in this context, it is used as sex-specific cue exploited by males (Estrada et al 2010).

A number of behavioural observations related to butterfly pheromones have been collected, and in some studies the composition of wings or glands have been analyzed, including some with electroantennogram data. However, few behavioural experiments have been done in order identify exact pheromone components. In *Eurema hecabe* L. (Lepidoptera: Pieridae) experimental evidence of male and female sex pheromone presence was demonstrated using behavioural experiments on wing extraction, but the pheromones were not identified (Takanashi et al 2001). Extractions from *Celastrina argiolus ladonides* L. (Lepidoptera: Lycaenidae) and subsequent GC-MS identification revealed that two lactone compounds, lavender lactone and δ-decalactone, induce responses from the butterfly. However, no behavioural experiments have been done (Hisashi et al 2013).

Already 40 years ago, the main components of *P. napi* wing secretion were identified as the two isomers of citral (a 1:1 mixture of neral and geranial) (Bergström and Lundgren 1973). Anderson et al (2007) clearly demonstrated the effect of citral in female mate acceptance in *P. napi*. Female acceptance of males increased when a higher concentration of citral was released. The Asian butterfly *Pieris melete* Ménétrières (Lepidoptera: Pieridae) also emits some unidentified sex pheromone from the wings (Kan and Hidaka 1997). Male sex pheromones have also been identified in the congeneric species *Pieris rapae* and *Pieris brassicae*. More than 120 components have been identified in solvent extracts of wings of these butterflies but only a few were related to male odour. The major components of *P. rapae* wing extract were ferrulactone, hexahydrofarnesylacetone and phytol; major components of *P. brassicae* were brassicalactone, hexahydrofarnesylacetone and phytol. Bioassays with males identified these lactones as components of the aphrodisiacs (Yildizhan et al 2009). By using deuterium labelled phytol it has been demonstrated that hexahydrofarnesylacetone is synthesized from phytol during the larval stage (Schulz et al 2011).

In 1976 Gilbert suggested that male *Heliconius* butterflies added some odour to females that made them unattractive to other males, and named it anti-aphrodisiac. After
more than 30 years the anti-aphrodisiac in *Heliconius* butterflies has finally been identified as β-ocimene, produced in adult butterflies from glucose (Schulz et al 2008). Andersson et al (2000) identified the anti-aphrodisiac in *P. napi* as methyl salicylate. In a later article they also demonstrated that methyl salicylate and indole are anti-aphrodisiacs in the congeneric *P. rapae*, and benzyl cyanide in *P. brassicae* (Andersson et al 2003). Using $^{13}$C carbon labelled molecules they demonstrated that methyl salicylate and benzyl cyanide are produced from phenylalanine ingested during the larval stage. The amount of female released anti-aphrodisiac decreased gradually after mating. The egg-parasitizing wasp *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) uses the anti-aphrodisiac released by mated female *P. brassicae*, i.e. benzyl cyanide, to locate their butterfly hosts. Interestingly, *T. brassicae* is equally attracted by mated females and males (Fatouros et al 2005). Moreover, benzyl cyanide induces a plant’s phytochemical response which attracts females of the egg parasitoid *T. brassicae* (Fatouros et al 2008). Indole, but not methyl salicylate, in the pheromone blend of *P. rapae* induces similar behavior in *T. brassicae* (Huigens et al 2009). This research group later demonstrated that *Trichogramma* wasps are more attracted to oviposition-induced plant cues than to indole, and that attraction to indole is a result of associative learning with *P. rapae* eggs (Pashalidou et al 2010, Huigens et al 2010). The release of the *P. brassicae* anti-aphrodisiac, benzyl cyanide, decreases as time goes by following mating, and so to the attraction of the parasitoids to the ovipositing females (Huigens et al 2011). All these recent data show possible trade-offs between reduction of mated female harassment, reduction of the risk of sperm competition and possible risk of parasitoid attacks.

Estrada et al (2011) suggested two models for the evolution of anti-aphrodisiacs under the influence of intra-sexual selection in butterflies. They analyzed chemical blends from abdominal glands in 11 *Heliconius* species, and suggested that all compounds in the blends from abdominal glands are anti-aphrodisiacs and utilize these data for their evolutionary model. Together these data demonstrated the importance of chemical cues in intra-specific interaction in the Lepidoptera suborder Rhopalocera.
1.5 Nutritional resources for butterflies

Butterflies utilize different nutritional resources during the larval and adult stages, but whether larval or adult derived nutrients are more important has been under discussion for a long time. In short, it has been assumed that resources derived from the larval stage are the more nutritionally rich and determines the fitness and longevity of the butterfly (Hill et al 1989, Romeis et al 2002). However, Mevi-Schutz and Erhardt (2005) suggested that butterflies can compensate a low nutrient intake during the larval stage with nutrition derived from adult feeding. Flower nectar is the most widespread adult food source for the majority of butterflies (Gilbert and Singer 1975).

Mevi-Schutz and Erhardt (2003) have shown that females of *Araschnia levana* L. (Lepidoptera: Nymphalidae) raised on a low-quality larval food showed a higher preference for flowers rich in nectar amino acids than females reared on rich amino acid media in the larval stage. Several studies have demonstrated the importance of adult uptake of sugars and water from flower nectar on fecundity (Norris 1934; Stern and Smith 1960; O’Brien et al 2000). Quantity and quality of adult food uptake directly affect longevity in *Papilio xuthus* L. (Lepidoptera: Papilionidae) butterfly (Watanabe 1992).

Baker and Baker (1973) discovered amino acids in floral nectar and later they demonstrated that flowers that are predominantly pollinated by butterflies have a higher concentration of amino acids (Baker and Baker 1975). Studies with radiotracers have demonstrated that amino acids ingested during the adult stage are incorporated into eggs (Boggs 1997). Females of *Inachis io* L. (Lepidoptera: Nymphalidae) (Erhardt and Rusterholz 1998) preferred flowers rich in amino acids over flowers lacking amino acids, but males of the same species showed no preference. Females of *P. napi*, that get a nuptial gift from the male during copulation, demonstrated declined interest in amino acid rich flowers with increasing mating frequency (Mevi-Schutz and Erhardt 2004). Production of this nuptial gift is physiologically costly as it consists of sperm, hormones, proteins, lipids, amino acids and water. Although some of the amino acids that males invest in the spermatophore are used by females for egg production some are also incorporated into female somatic tissue (Boggs and Gilbert 1979; Boggs 1981; Wiklund et al 1993; Wedell 1996). Finally, Cahenzli and Erhardt, in 2013, demonstrated that
amino acids enhance reproduction and fitness of male *Coenonympha pamphilus* (Lepidoptera: Nymphalidae).

Most nitrogen in butterflies is incorporated during the larval stage and ability to restore nitrogen in the adult stage is limited (O’Brien et al 2002). Although multiple copulations increase longevity and fecundity of females, the females need to relocate nitrogen from the thorax for egg production and, similarly, males need to transfer nitrogen from the thorax for spermatophore production (Stjernholm and Karlsson 2000).

O’Brien et al (2002) claimed that females of Lepidoptera have the ability to synthesize egg amino acids from nectar resources, but are not able to utilize non-essential amino acids during the adult stage. Later they demonstrated that *Heliconius charitonia* L. (Lepidoptera: Nymphalidae) are able to utilize essential amino acids from pollen.

The ability of butterflies to utilize essential amino acids from nectar to produce pheromones has been unclear and no evidence has been collected. Andersson et al (2000) have shown the production of anti-aphrodisiac from phenylalanine during the larval stage, but no data are available on anti-aphrodisiac production in adult butterflies, nor how butterfly males utilize resources from flower nectar. Also, no information has been available about alternative materials that butterflies can utilize if suffering from amino acid deficiency during the adult stage. Moreover, no information has been collected regarding differences or similarities in pheromone production in bivoltine butterflies (two generations during one year). The two generations are under different selection pressures and both behavioural and physiological seasonal polyphenisms have been shown previously.

1.6 Production of aromatic volatiles in flower fragrance

Flower scent consists of a complex bouquet of volatiles, the concentration and composition of which determine the main characteristics of flowers’ fragrance. The most common chemicals involved in flower scent are benzenoids, terpenoids, fatty acid derivatives and indole compounds (Knudsen and Tollsten 1993).

Benzenoids are one of the most widespread groups of volatiles in flower scent, responsible for pollinator attraction and other ecological functions. The main substrate for benzenoids in plants is the amino acid phenylalanine, and by providing plants with
this substrate it is possible to alter floral fragrance even without gene modification. 2-Phenylethanol is one of the most widespread aromatic compounds in flower fragrance. Biosynthesis of 2-phenylethanol in Petunia hybrida is catalyzed by phenylacetaldehyde synthase (PAAS) (Kaminaga et al 2006). This enzyme has strict specificity for phenylalanine and catalyzes the stoichiometric oxidative decarboxylation. Phenylacetaldehyde is also biosynthesized in roses from phenylalanine with pyridoxal-5’-phosphate-dependent L-aromatic amino acid decarboxylase (AADC) and phenylacetaldehyde was subsequently converted to 2-phenylethanol by the action of phenylacetaldehyde reductase PAR (Ziyin et al 2009).

Common biosynthesis of benzyl acetate in plants proceeds via cinnamic acid, synthesized by phenylalanine ammonia-lyase from L-phenylalanine. Cinnamic acid is then converted to benzaldehyde and benzylalcohol, although the enzymes responsible for this transformation are still unknown. Benzyl acetate is synthesized from benzylalcohol via CoA:benzylalcohol acetyltransferase (Pichersky et al 2007).

In the plants MeS is synthesized from phenylalanine via PAL and via decarboxylation of trans-cinnamic acid to benzoic acid and its subsequent 2-hydroxylation to salicylic acid (Lee 1995; Boatright et al., 2004) followed by methylation to MeS (Effmert et al., 2005), however no experimental data showing incorporation of labelled atoms from L-phenylalanine in methyl salicylate are available. Wildermuth et al (2001) have shown that in Arabidopsis thaliana (L.) Heynh. (Brassicales: Brassicaceae) salicylic acid is synthesized from chorismate by means of isochorismate synthase revealing another biosynthetic pathway. Salicylic acid can afterwards be methylated to methyl salicylate by carboxyl methyltransferase (Wildermuth, 2006) Fig. 1.

In our study we demonstrated a method to trace incorporation of benzenoids from flower fragrance to pheromone components in the adult butterflies, as well as how to alter volatile composition of flower fragrance.
Figure 1. Hypothetic scheme of the biochemical pathways of aromatic volatiles found in floral scents of *Bunias orientalis* and *Alliaria petiolata* flower fragrance. Partly based on Wildermuth, 2006; Dudareva et al 2013. Solid lines show reactions with identified enzymes and dashed lines with unidentified enzymes. 1) Phenylalanine, 2) Phenyl acetaldehyde, 3) 2-Phenylethanol, 4) Cinnamic acid, 5) Benzaldehyde, 6) Benzylalcohol, 7) Benzyl acetate, 8) Benzoic acid, 9) Methyl benzoate, 10) Salicylic acid, 11) Methyl salicylate, 12) Cinnamaldehyde, 13) Cinnamylalcohol, 14) Acetophenone, 15) Benzyl cyanide, 16) Shikimic acid, PAL phenylalanine ammonia lyase, PAAS phenylacetaldehyde synthase, BSMT benzoic acid/salicylic acid carboxyl methyltransferase, PAR phenylacetaldehyde reductase, BALDH benzaldehyde dehydrogenase, BEAT cetyl-coenzyme A:benzyl alcohol acetyltransferase, SAMT salicylic acid carboxyl methyltransferase.
2. Materials and Methods

2.1 Butterflies
The Green-veined white, *Pieris napi* L. (Lepidoptera: Pieridae) butterflies were reared at the Department of Zoology, Stockholm University. The founders of the laboratory population were collected in the vicinity of Stockholm, Sweden. After being caught females were taken to the laboratory where they laid eggs on their host plants *Alliaria petiolata* M.Bieb (Brassicales: Brassicaceae) and *Armoracia rusticana* G.Gaertn., B.Mey. and Scherb. (Brassicales: Brassicaceae). In papers I and II, larvae were reared in two different light regimes in order to induce direct (20:4 h light/dark) and diapause (12:12 h light/dark) development. In papers III and IV, larvae of *P. napi* were fed on *A. petiolata* under laboratory conditions at a 22:2h LD photoperiod and 25 °C and the larval development time was 14±2 days.

2.2 Flowers
Inflorescences of Turkish Rocket, *Bunias orientalis* L. (Brassicales: Brassicaceae) and Garlic Mustard, *Alliaria petiolata* M.Bieb. (Brassicales: Brassicaceae) were collected in the surroundings of the Royal Institute of Technology (KTH, Stockholm, Sweden, 59.353° N 18.068° W) between 6 and 8 pm during May and June. Flowering stems were cut with scissors and put in water immediately.

2.3 Collection of head space volatiles, solid phase microextraction (SPME)
Solid phase microextraction (SPME) (Fig. 2) is a successfully used method to collect headspace volatiles for plants and insects (Borg-Karlson and Mozuraitis 1996, Moneti et al 1999, Augusto and Valente 2002, Schäfer et al 1995, Tholl 2006, Richter and Schellenberg 2007). It is an easy and sensitive method for sampling of volatiles from complex matrices (Pawlisyn 2009). In contrast to conventional exhaustive extraction methods, SPME is based on the partition equilibrium of the analytes between the sample matrix and the extraction phase (Fang 2013). In general, quantification is the major problem in SPME, especially in *in vivo* sampling. SPME methods are sensitive to temperature, thus it is better use SPME at a constant temperature normally around 20 °C.
Since our work is focused on the collection of insect and flower volatiles, the conditions for the butterflies and plants should be as natural as possible, at relevant temperatures and light conditions. All these factors can conceivably influence the results (Hakola et al 2006, Kesselmeier 1999). Time of sample extraction also influences the results (the butterfly conditions and plant flower circadian rhythm), and in addition longer time adsorption favours absorption of larger molecules. Thus in our experiment extraction time was generally set to 30 minutes for detached and crushed abdomen of butterflies and 1 hour for trapping the flower volatiles.

There are number of different fibres commercially available to facilitate extraction of diverse compounds. The mixed coating polydimethylsiloxane/divinylbenzene (PDMS/DVB) was suitable for the volatiles in our experiments.

![Solid phase microextraction (SPME) tool](image)

**Figure 2.** Solid phase microextraction (SPME) tool. (A) General view, (B) Cross section. Based on (Mozuraitis 2000)

### 2.4 Sampling technique

To collect the odours of butterflies after various experiments and treatments, butterflies were first stored in the freezer at -18 °C. Defrosted abdomens immediately start to release volatiles; thus, dissection to obtain the spermatophore was not a sufficient method to analyse the spermatophore volatile composition. When volatile profiles from spermatophores coincided with the compounds found in abdomen, we decided that it was
possible to use the abdomen and it could be individually placed in a 1.7 ml glass vial, and crushed. The volatiles were collected by SPME, followed by, in certain cases, solvent extraction of the abdomen with hexane. A purified SPME fibre (65 µm polydimethylsiloxane/divinylbenzene) was used and volatiles released were collected for 30 min. To identify odours emitted by live females, a glass cylinder (height 18 cm, diameter 8 cm), with one small opening for inserting a SPME needle was used (Andersson et al 2000). Two females were placed in the glass cylinder and on average one female flew while the other female released volatiles in response to the flying butterfly. Sampling lasted 1 hour. For collecting the volatiles produced in the androconia, the four wings of each individual male were detached and placed intact in a 1.7-ml glass vial. 200 µl hexane (spiked with 0.08 mg/ml n-pendadecane as internal standard) was added, and extraction of the wings was done for 12 hrs at room temperature (Paper I and II).

During collection of the flower emissions, flowers were kept in a glass cylinder (height 11 cm, diameter 4.5 cm) that was sealed with aluminium foil. Inflorescences of B. orientalis were immersed in 0.1% of non-labelled L-phenylalanine water solution. From the multiply branched raceme of each of the inflorescences, one of the smaller racemes (containing a subset of flowers) was cut and put in tap water, serving as a control. The A. petiolata inflorescences collected for the experiments had only one central raceme; therefore, plants growing in the immediate vicinity of the plants that were experimentally treated were collected and used as controls. Flowers were kept at 20-22 °C for 16 hours with natural light (III).

2.5 Separation and identification
Separation and identification of the butterfly and flower volatiles was made using a Varian 3400 Gas Chromatograph (GC) coupled to a Finnigan SSQ 7000 Mass Spectrometer (MS). GC-MS is especially suitable for analysing small and thermally stable molecules such as volatiles (Angerosa et al 1995). Separation of the mixture of compounds in the GC column is based on different affinities of the compounds in stationary phase and temperature gradient. Different compounds elute at different times from the column and this time is named “retention time”. Commercially available
columns differ in their length, diameter, film thickness and stationary phase properties. A DB-WAX column (internal diameter 0.25 mm, film thickness 0.25 μm and length 30 m; J & W Scientific, Folsom, USA) was used in our experiment. Identification of separated compounds was made by comparison of their retention times and mass spectra with those of authentic reference standards as well as comparing mass spectral data with those available from the NIST mass spectral library, programme version 2.0 (National Institute of Standards and Technology, USA).

2.6 Ratio of isotope incorporation
The incorporation of $^{13}$C$_6$ glucose was determined by selecting characteristic ions representing the labelling of the compounds. By calculating the proportion of the ions 71 (labelled) to 69 (non-labelled) the incorporation during the first 48 hours of development after eclosure could be measured (Paper II).

The level of incorporation of $^{13}$C and $^2$H in fertilizing and feeding experiments (Papers III and IV) was determined as the ratio between the labelled molecular ion ($M_{+n}$) and the non-labelled ($M_+$) molecular ion in compounds of treated samples minus the ratio of the molecular ions ($M_{+n}$) and the molecular ions ($M_+$) of compounds in control (non-labelled) samples.

$$\text{Ratio of incorporation} = (M_{+n}/M_+)_\text{treated} - (M_{+n}/M_+)_\text{control}$$

2.7 Statistical analysis of data
Statistical analysis was performed in R (Version 2.15.1, 2012) or in STATISTICA 10. If the data met parametric test assumptions T-paired, or ANOVA tests were used, otherwise Wilcoxon signed-rank test, Mann–Whitney U test or Kruskal–Wallis were applied.

3. Results

3.1 Aphrodisiac production in *Pieris napi*

*Paper I*
3.1.1 Aphrodisiac production in direct and diapause generations

There was a distinct difference between directly developing and diapause generation males in the proportion of male sex pheromone citral (neral and geranial 1:1 mixture) extracted from wings 4, 6, 8 and 24 h after eclosion (Fig. 3). Diapause generation males emitted some of the adult scent citral already 4 h after eclosure and there was no effect of age on the proportion of the citral ($\chi^2 = 5.88$, df=3, p=0.12). For directly developing males, there was a significant effect of age ($\chi^2 = 15.58$, df=3, p=0.001); the wings from the younger age classes had almost no neral and gernaial but had high proportions of other volatiles such as geraniol, geranic acid and nerol, we suggest that these compounds are precursors of the final male sex pheromone blend of neral and geranial.

[Graph showing emission of citral by diapausing and directly developing males 4, 6, 8 and 24 h after eclosure. Proportion of citral of emitted substances (%), mean±SE, consisting of neral (dotted) + geranial. In young males, there is a difference between the generations in emission of citral.]

3.1.2 Male mating propensity

The majority of males (direct 100%, diapause 91%) did not mate until the second day of the experiment, the time to mating for these males includes the 16 h of inactivity imposed by the light regime (Fig. 4). Males from the diapause generation (n=22) mated sooner
(times hereafter given as mean±SE), 1383±80 min, after eclosion than males from the directly developing generation (n=17), 1771±114 min (ANOVA, $F_{1,37}=8.27$, $p=0.007$).

Males from the directly developing generation eclose less sexually mature than diapause generation individuals (Fig. 3). This results in a longer period before mating that coincides with the production of neral and geranial.

**Paper II**

3.1.3 The volatiles of the wing scent in direct and diapause generations

The results of this study also show that males of the directly-developing generation do not emit adult scent composition at eclosion but have more of the corresponding alcohols early in life, indicating that males in the directly-developing generation eclose less sexually mature than diapause generation individuals. The volatiles of the wing scent consisted of geraniol, nerol, geranial, and neral in variable amounts depending on the age and generation of the butterflies (Fig. 5, 6). At 4 hrs after eclosure, none of the directly-developing males, in contrast to the diapause-generation males, had any measurable amount of neral, and only one out of four had any geranial. The proportion of citral (geranial + neral) in the scent composition (alcohols + aldehydes) differed between generations during the first 6 hrs of adult life (at 4 hrs, Mann–Whitney U test, $U=1$,
P=0.02; at 6 hrs, Mann–Whitney U test, U=4, P=0.03). After 8 and 24 hrs, the difference in sex pheromone composition had disappeared, and the generations no longer differed in the proportion of citral (8 hrs, Mann–Whitney U test, U=28, P=0.77; 24 hrs, Mann–Whitney U test, U=27, P=0.2). Directly-developing males could be considered mature 24 hrs after eclosure, since the scent composition no longer changed with age and citral proportion (amount neral + geranial out of total scent) was, at 24 hrs (median; 89 % citral), not significantly different from one-week-old males (median; 93 % citral) (Mann–Whitney U test, U=8, P=0.4). One out of the five diapausing pupae had geraniol present in small amounts approximately 1 hr before eclosion. None of the ten directly-developing pupae had traceable amounts of any of the four wing scent components.

![Figure 5](image_url)

**Figure 5.** Relative amounts of scent composition of the four major compounds following eclosure. (A) Directly-developing *P. napi* males; (B) Diapausing *P. napi* males
Figure 6. Total ion chromatograms of scent composition of the four major compounds 4 and 24 hours after eclosure in *P. napi* males.

Figure 7. Adult $^{13}$C glucose feeding. Proportion of labelled to non-labelled fragments (71/69) in the four components of *P. napi* wing scent. Average ± SE.
Figure 8. Representative mass spectra for the four wing scent compounds from *P. napi* male wing after 48 hrs of feeding on $^{13}$C$_6$-labelled glucose (left column). Non-labelled mass spectra of the compounds to the right.
3.1.4 Larva and adult $^{13}$C glucose feeding

From our experiments with $^{13}$C-labelled glucose, we showed that male *P. napi* can synthesize geranial and neral from both larval (not shown) and adult-derived glucose (Fig. 7, 8). Larval feeding: Labeled $^{13}$C from fully labelled glucose ingested in the larval stage, showed up in geraniol, nerol, geranial, and neral in the adult stage as measured by volatiles emitted. Around half of the emitted substances 6 hrs after eclosure were derived from the labeled carbon. The average proportion ± SE of labeled to non-labelled wing scent components increased significantly with the time allowed for foraging. For the proportion of the selected ions 71\69 (Fig. 8); Spearman’s rank correlation, geranial $\rho=0.83$, $P<0.001$; geraniol $\rho=0.82$, $P<0.001$; neral $\rho=0.62$, $P<0.001$; nerol $\rho=0.87$, $P<0.001$. Interestingly, when comparing the proportion of the ion fragments 71\69, a clear time difference in incorporation of $^{13}$C was observed between the compounds. Incorporation of $^{13}$C into geranial and neral appeared later than incorporation of $^{13}$C into the corresponding alcohols. Already after 4 hrs of feeding on labelled glucose, labelled geraniol and nerol were produced (Fig. 7). Labelled geranial appeared after 8 hrs, and labelled neral was detected only after 32 hrs of adult feeding (Fig. 7).

**Paper III**

3.2 Fertilizing plants with L-phenylalanine

Since *P. napi* males utilize phenylalanine as a substrate for producing the anti-aphrodisiac, we wished to enhance the production of certain benzenoids present in flower scent by adding the aromatic amino acid L-phenylalanine to the plant. GC-MS analysis revealed a wide range of benzenoid compounds from inflorescences of two species from the Brassicaceae family: *Bunias orientalis* and *Alliaria petiolata.*

*A. petiolata* flowers released 10 benzenoid compounds including formanilide, benzaldehyde, methyl benzoate, benzyl cyanide and formotoluidide each of them exceeding 10% of the total blend trapped on the fibre. In addition, *A. petiolata* released methyl salicylate, the major component of the anti-aphrodisiac pheromone of *P. napi.* Treatment of the inflorescences with the 0.1% solution of L-phenylalanine resulted in an
increased amount of acetophenone, 2-phenylethanol and benzylalcohol. Formotoluidide, 4-metoxybenzaldehyde, and formanilide were released in significantly lower relative amounts compared with those collected from control samples (Fig. 9). 4-Metoxybenzaldehyde has an incorporation of $^{13}$C L-phenylalanine while formotoluidide and formanilide had not. Surprisingly, methyl salicylate was not labelled in any individual plants; it has been assumed for a long time that methyl salicylate is produced from phenylalanine (Lee et al 1995). All other benzenoids demonstrated incorporation of labelled phenylalanine (Fig. 10).

The floral bouquets of untreated *B. orientalis* plants were comprised of 9 benzenoid compounds; among them 2-phenylethanol, phenylacetaldehyde, and benzylalcohol together contributed 90% of the total amount of fragrance (Fig.11). Relative amounts of 2-phenylethanol increased while methyl benzoate decreased significantly when plants were treated with 0.1% solution of phenylalanine in water compared with control plants (Fig. 11). The two plant species showed significant changes in the composition of flower scent after being provided with L-phenylalanine. The major increase was found for 2-phenylethanol. This coincided with the high incorporation in $^{13}$C in 2-phenylethanol in *B. orientalis* and *A. petiolata*.

To mimic the natural situation for the nectar feeding butterflies, volatiles were trapped from abdomens of mated males that were allowed to feed on flowers in the 1% labelled L-phenylalanine water solution. Incorporation of $^{13}$C in methyl salicylate, benzyl cyanide and 2-methoxyphenol was found which confirms that adult males are able to acquire precursors of their anti-aphrodisiacs by feeding on flowers and produce aromatic compounds that to a certain extent may restore their titer of anti-aphrodisiacs (Table 1).
Figure 9. Effect on *Alliaria petiolata* floral fragrance after 0.1% fertilization with L-phenylalanine. Vertical bars represent standard error; stars indicate that amounts of compound in control and treated samples differ significantly.

Figure 10. Effect of 0.05% \(^{13}\text{C}\)-labelled [L-ring-\(^{13}\text{C}\)] phenylalanine incorporation in *Alliaria petiolata* flower fragrance. Vertical bars represent standard error.
**Figure 11.** Effect on *Bunias orientalis* floral fragrance after 0.1% fertilization of non-labelled L-phenylalanine. Vertical bars represent standard error; stars indicate that amounts of compound in control and treated samples differ significantly.

**Figure 12.** Effect of 0.05 % $^{13}$C-labelled [L-ring-$^{13}$C] phenylalanine incorporation in *Bunias orientalis* flower fragrance. Vertical bars represent standard error.
Table 1. Ratio of labelled and non-labelled molecular ions in the flower scent constituents after being fertilized with 1% labelled phenylalanine and P. napi male volatiles after feeding on these flowers.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Bunias orientalis, N = 2</th>
<th>Male abdomen, N = 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylacetaldehyde</td>
<td>10.5 (1)</td>
<td></td>
</tr>
<tr>
<td>2-Phenylethanol</td>
<td>11.9 (0.3)</td>
<td></td>
</tr>
<tr>
<td>Methyl phenylacetate</td>
<td>9.6 (0.08)</td>
<td></td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>4 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Benzylalcohol</td>
<td>41.3 (34.5)</td>
<td></td>
</tr>
<tr>
<td>Methyl benzoate</td>
<td>3.2 (3.2)</td>
<td></td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>0.5 (0.3)</td>
<td></td>
</tr>
<tr>
<td>Cinnamylalcohol</td>
<td>1.8 (1.4)</td>
<td></td>
</tr>
<tr>
<td>Methyl salicylate</td>
<td>0.03 (0.001)</td>
<td></td>
</tr>
<tr>
<td>2-Methoxyphenol</td>
<td>0.02 (0.008)</td>
<td></td>
</tr>
<tr>
<td>Benzyl cyanide</td>
<td>0.01 (0.005)</td>
<td></td>
</tr>
</tbody>
</table>

N is the number of replicates; numbers in brackets represent standard error; nl means not labelled, pi means possible incorporation.

Paper IV

3.3 Production of anti-aphrodisiac and related male compounds in adult butterflies

Feeding butterflies with ring $^{13}$C$_6$ labelled L-phenylalanine showed incorporation of $^{13}$C atoms in methyl salicylate, benzyl cyanide and 2-methoxyphenol. Thus it is clearly demonstrated that males of P. napi are able to produce anti-aphrodisiacs and other aromatic compounds in male sex pheromones even in the adult stage. Abdomens from females mated with these males, contained $^{13}$C labelled methyl salicylate, benzyl cyanide and 2-methoxyphenol; hence, this experiment demonstrates that, via the spermatophore, males transfer aromatic compounds produced during adult stage (Table 2).

After showing the incorporation of labelled $^{13}$C into anti-aphrodisiac by nectar feeding butterflies, a new group of males were fed with deuterium labelled synthetic compounds common in flower volatiles. The results showed that methyl salicylate and 2-methoxyphenol were produced from all labelled substrates except from 2-phenylethanol, while benzyl cyanide originated only from L-phenylalanine (Table 3).
Table 2. Ratio of labelled and non-labelled molecular ions of aromatic compounds in male *Pieris napi* abdomen measured by MS, after feeding with labelled substrates.

<table>
<thead>
<tr>
<th><em>P. napi</em> biosynthesized compounds</th>
<th><em>P. napi</em> females mated with males fed with labelled</th>
<th>Females fed labelled L-Phe, mated with males not fed labelled L-Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-Phe Abdomen N = 8 Live N = 5</td>
<td>Abdomen N = 3</td>
</tr>
<tr>
<td>Methyl salicylate</td>
<td>0.9 (0.6) 0.9 (0.2) 0.4 (0.02) 0.8 (0.07) 0.9 (0.1)</td>
<td>nl</td>
</tr>
<tr>
<td>2-Methoxyphenol</td>
<td>0.5 (0.4) pi 0.03 (0.04) nl pi nl</td>
<td>nl</td>
</tr>
<tr>
<td>Benzyl cyanide</td>
<td>0.9 (0.5) pi nl nl nl nl nl</td>
<td>nl</td>
</tr>
</tbody>
</table>

N is the number of replicates; numbers in brackets represent standard error; nl means not labelled; pi means possible incorporation; L-Phe represents L-Phenylalanine.

Table 3. Ratio of labelled and non-labelled ions of aromatic compounds in male *P. napi* abdomen measured by MS, after feeding with labelled substrates.

<table>
<thead>
<tr>
<th><em>P. napi</em> biosynthesized compound</th>
<th>Benzaldehyde N=5</th>
<th>Benzy alcohol N=7</th>
<th>Benzoic acid N=2</th>
<th>Methyl benzoate N=3</th>
<th>Benzy cyanide N=5</th>
<th>Methyl cinnamate N=5</th>
<th>2-Phenyl ethanol N=6</th>
<th>Phenylalanine N=9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl salicylate</td>
<td>0.4 (0.08)</td>
<td>0.7 (0.1)</td>
<td>0.2 (0.07)</td>
<td>0.2 (0.03)</td>
<td>0.2 (0.07)</td>
<td>0.4 (0.04)</td>
<td>nl</td>
<td>0.4 (0.06)</td>
</tr>
<tr>
<td>2-Methoxyphenol</td>
<td>0.4 (0.06)</td>
<td>0.4 (0.09)</td>
<td>0.2 (0.06)</td>
<td>0.1 (0.03)</td>
<td>0.1 (0.04)</td>
<td>0.3 (0.04)</td>
<td>nl</td>
<td>0.2 (0.05)</td>
</tr>
<tr>
<td>Benzyl cyanide</td>
<td>nl</td>
<td>nl</td>
<td>nl</td>
<td>nl</td>
<td>*</td>
<td>nl</td>
<td>nl</td>
<td>0.6 (0.1)</td>
</tr>
</tbody>
</table>

Numbers in brackets represent standard error. * means not measured due to possible contamination, nl represents not labelled.
4. Conclusions

Aphrodisiac concentration, production and maturity of Pieris napi males depend on whether they have gone through direct or diapause development.

Both aphrodisiac and anti-aphrodisiac pheromones in P. napi are produced from resources ingested in the larva stage and from nutrients consumed in the adult stage.

Males of P. napi produce aphrodisiac from glucose and anti-aphrodisiac. from both L-phenylalanine and from common flower fragrance constituents.

Fertilizing plants with stable isotopes is an effective method to investigate the transfer of pheromone building blocks from flowering plants to butterflies.

Fertilizing plants with amino acids, like L-phenylalanine, is an effective method to alter flower fragrance.

Methyl salicylate is not biosynthesized from L-phenylalanine in flowers of Alliaria petiolata.
5. Acknowledgements

First of all I express my thanks to my supervisors Raimondas Mozuraitis, Anna-Karin Borg-Karlson and Christer Wiklund for accepting me as a PhD-student, for involving me in an interesting project and for sharing your knowledge, enthusiasm, discussions and support during all these years.

To my collaborators Helena Larsdotter-Mellström, Magne Friberg and Ilme Libikas, it was a pleasure to work with you! And Mikael Palm, that I have never seen personally, but whose input in my first project is invaluable. Thank you all for nice collaboration, for answering all my questions and for your patience.

I would thank all my colleagues in the Chemical Ecology group. To Karolin Axelsson, who showed me spermatophore dissection. Astrid Kännaste who taught me flower emission sampling. Tao Zhao who indeed helped me the first time with Gas-Chromatography and Mass-Spectra analysis. Azeem Muhammad who was always ready to answer my chemistry questions. Nancy Cabezas and Lina Lundborg for all the fun that we shared together. Katinka Pålsson who helped me with statistical analysis and always made fun in the room with ”hunger” and “headache”. To all my other colleagues in the group.

Thanks to the rest of the Organic Chemistry Division, to “Chinese”, “Spanish”, and “German” mafias for nice lunches in the kitchen and great Christmas dinners. I give thanks to PhD pub at KTH that always reminded me that I have “the funniest and the sexiest project at the institute” and after some hard days working told me that “Rusha, is the girl who study sex in butterfly”.

To my neighbours in Lappis that “study hard and party harder”, to Ali Imran, Azadeh Nilchian (the most “intelligent” and “educated” person in the corridor), Özlem Seçkiner, Jędrek Burszta and all others.

The extraordinary people I met in Stockholm such as Elchin, Elena, Oleksandr, Vardan, Anna, Diana, Keshav and all the others. Thank you my friends, you made my life better and brighter.

To Andrii who always supports me and for his patience. I am deeply grateful to my parents Nuriya and Nuris, I hope that I make you proud. To my sister and brother in
low Evgeniy who always helped me when I asked. To my cutest nephews Daniel, who in 4 years was able to pronounce “Lepidoptera” and know that is a “butterfly” and to Michael. And to the baby boy that was with me during my entire thesis writing. Thank you, you are really kind to your mam and made the last year of my PhD the happiest.
6. References


7. Appendix A: contribution

The following is a description of my contribution to the Publication I and IV, as requested by KTH

**Paper I.** I participated in the planning and performed partly the chemical analysis of the male sex pheromone and did statistical analysis of the chemical data.

**Paper II.** I participated in the planning of experiments, performed chemical analyses and did statistical analysis of the chemical data.

**Paper III.** I participated in the planning of experiments, performed chemical analyses and did statistical analysis of the chemical data and wrote the manuscript.

**Paper IV.** I participated in the planning of experiments, performed chemical analyses and did statistical analysis of the chemical data and wrote the main part of the manuscript.
8. Appendix B: Chemical structures of pheromone components in three *Pieris* species

<table>
<thead>
<tr>
<th>Species name</th>
<th>Aphrodisiac</th>
<th>Anti-aphrodisiac</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P.napi</em></td>
<td>Geranial</td>
<td>Methyl salicylate</td>
</tr>
<tr>
<td></td>
<td>Neral</td>
<td></td>
</tr>
<tr>
<td><em>P.rapae</em></td>
<td>Ferrulactone</td>
<td>Methyl salicylate</td>
</tr>
<tr>
<td></td>
<td>Hexahydrofarnesylacetone</td>
<td>Indole</td>
</tr>
<tr>
<td></td>
<td>Phytol</td>
<td></td>
</tr>
<tr>
<td><em>P.brassicae</em></td>
<td>Brassicalactone</td>
<td>Benzyl cyanide</td>
</tr>
<tr>
<td></td>
<td>Hexahydrofarnesylacetone</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phytol</td>
<td></td>
</tr>
</tbody>
</table>