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Gene families and antennal morphology

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DEPARTMENT OF BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY

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Title and subtitle: Evolution of olfaction in Lepidoptera and Trichoptera – Gene families and antennal morphology

Abstract

In moths, females produce sex pheromone compounds to attract males over a long distance for mating. The antennae of moths and many other insects have specialized odorant receptors (ORs), called pheromone receptors (PRs), to sense the pheromone compounds and they group in a monophyletic clade (PR clade).

In this thesis, I investigated and compared various components of the olfactory system in different species of Trichoptera and Lepidoptera (moths and butterflies). I made an effort to particularly understand the origin of the PR clade, the pheromone binding proteins (PBPs) and other chemosensory genes, differences in antennal morphology, presence of Macro glomerular complex (MGC). I used a variety of experimental approaches ranging from microscopy studies, next-generation sequencing and *in vitro* functional characterization of receptors.

Eriocrania semipupurella (Eriocraniidae: Lepidoptera) is more basal among the moths than *Lampronia capitella* (Prodoxidae: Lepidoptera). However, *L. capitella* is the most basal moth species using Type I pheromone compound. I functionally characterized three receptors from *E. semipupurella*, two of them responding to primitive pheromone compounds (Type 0 pheromone compounds) and structurally similar plant volatiles, indicating that these receptors likely have evolved from common plant volatile-detecting ORs. One receptor positioned at the base of the conserved pheromone receptor (PR) clade selectively responded to a plant volatile β-caryophyllene, which suggests that PRs of derived moths may also have evolved their function from plant volatile detecting ORs. In addition, a *L. capitella* specific clade of ORs falls in between the classical PR clade and the β-caryophyllene receptor. The functional activity of three *L. capitella* ORs, that responded to Type I sex pheromone compounds, suggests that the PR clade can be expanded with these receptors.

The antennal transcriptome analysis provided the first set of chemosensory gene families from Trichoptera and basal Lepidoptera. Furthermore, the *L. capitella* transcriptome comprised chemosensory genes that group within the PR and PBP clades, which contain specialized proteins involved in sex pheromone detection so far only reported in more derived, so-called ditrysian moths. These findings suggest that specialized chemosensory proteins have evolved in parallel with the transition of different sex pheromone types in Lepidoptera.

Antennal morphology studies revealed that there was a shift in the major sensilla type, from sensilla auricillica in Trichoptera to sensilla trichoidea in derived Lepidoptera. Preliminary results from immunocytochemistry studies of antennal lobes show the presence of MGC-like structures in male *E. semipupurella* and both sexes of *R. nubila* which possibly are homologous to MGCs of derived moth. On the other hand, the MGC is present only in male AL of *L. capitella* which may correspond to detection of female-produced pheromone compounds by the male. This is in line with what previously was shown in derived moths that pheromone detecting neurons of sensilla trichoidea project into MGC and that these enlarged glomeruli are dimorphic and mostly present in males. Interestingly, in the butterfly *Bicyclus anynana* the MGC-like glomeruli seem to present only in female AL. In addition, the number of ORs found in the antennal transcriptome roughly correspond to the number of glomeruli's found in the antennal lobes of *R. nubila*, *E. semipurpurella* and *L. capitella*.

My work on olfaction in Trichoptera and primitive Lepidoptera has demonstrated that (1) receptors involved in detection of Type 0 and I pheromone compounds have possibly evolved independently from different plant volatile detecting ORs, (2) the functional studies of *L. capitella* PRs add functional support to the PR clade, and (3) some Lepidoptera specific chemosensory proteins are only present in *L. capitella* which use Type I pheromone for sex communication. This illustrates that the chemosensory gene families, at least at the level of antennal expression may be associated with different pheromone types. (4) Similarly, antennal morphology studies show a shift in major types of olfactory sensilla, from sensilla placodea in basal moths to sensilla trichoidea in derived moths.

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Jothi Kumar Yuvaraj

To my lovely mother Shanthi Marappan

Content

List of Papers

This thesis is based on the following papers,

I. **Yuvaraj JK**, Corcoran JA, Andersson MN, Newcomb RD, Anderbrant O, Löfstedt C. 2017. Characterization of odorant receptors from a non-ditrysian moth, *Eriocrania semipurpurella* sheds light on the origin of sex pheromone receptors in Lepidoptera. *Molecular Biology and Evolution*, msx215, https://doi.org/10.1093/molbev/msx215.

II. **Yuvaraj JK**, Andersson MN, Corcoran JA, Anderbrant O, Löfstedt C. 2017. Functional characterisation of odorant receptors from *Lampronia capitella* adds functional support for the pheromone receptor clade in Lepidoptera. *Manuscript.*

III. **Yuvaraj JK**, Andersson MN, Zhang DD, Löfstedt C. 2017. Antennal transcriptome analysis of chemosensory gene families in Trichoptera and basal Lepidoptera. *Manuscript.*

IV. **Yuvaraj JK**, Andersson MN, Anderbrant O, Löfstedt C. 2017. Diversity of olfactory structures: a study of antennal sensilla in relation to olfactory cues in Trichoptera and Lepidoptera. *Manuscript.*

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Authors contributions to the papers

I.   J.K.Y., J.A.C, M.N.A., O.A., R.D.N. and C.L. conceived and designed the study. J.K.Y and O.A. collected the biological material. J.K.Y. and M.N.A performed transcriptomic data analysis and constructed the phylogenetic tree in fig. 3. J.K.Y and J.A.C. performed molecular work, cell line generation and culturing. J.K.Y performed functional assays. J.K.Y. drafted the manuscript with contributions from M.N.A., J.A.C., O.A., R.D.N. and C.L. All authors read and approved the final version of the manuscript.

II.   J.K.Y., M.N.A., J.A.C., O.A. and C.L. conceived and designed the study. J.K.Y collected biological material, performed molecular work, and functional experiments. J.K.Y., and M.N.A. performed transcriptome data analysis and constructed the phylogenetic tree. J.K.Y. and J.A.C. performed cell line generation and cell culturing. M.N.A. conducted statistical analyses. J.K.Y. and M.N.A. together wrote the manuscript with contributions from J.A.C., O.A. and C.L. All authors read and approved the final version of the manuscript.

III.   J.K.Y., M.N.A., and C.L. conceived and designed the study. J.K.Y collected biological material. J.K.Y. performed molecular work with assistance from D.D.Z. J.K.Y. and M.N.A. performed transcriptome data analysis and constructed the phylogenetic trees. J.K.Y. and M.N.A wrote the manuscript together with contributions from D.D.Z., and C.L. All authors read and approved the final version of the manuscript.

IV.   J.K.Y., M.N.A., O.A. and C.L. conceived and designed the study. J.K.Y collected biological material and prepared the samples for microscopy. J.K.Y performed scanning and transmission electron microscopy imaging. J.K.Y. wrote the manuscript with contributions from M.N.A., O.A., and C.L. All authors read and approved the final version of the manuscript.

Abbreviations

AL- Antennal lobe

BGI- Beijing genomics institute

CI- Confidence interval

EC50- Half maximal effective concentration

GOBP- General odorant binding protein

HEK cells- Human embryonic kidney cells

IR- Ionotropic receptor

MGC- Macro glomerular complex

OBP- Odorant binding protein

ODE- Odorant degrading enzyme

OR- Odorant receptor

ORCO- Odorant receptor co-receptor

OSN- Olfactory sensory neurons

PBP- Pheromone binding protein

PCR- Polymerase chain reaction

PR- Pheromone receptor

PR clade- Pheromone receptor clade

RACE- Rapid amplification of cDNA ends

SEM- Scanning electron microscopy

SNMP- Sensory neuron membrane protein

TEM- Transmission electron microscopy

TREx- Transcriptional Repressor

Abstract

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On the other hand, the MGC is present only in male AL of *L. capitella* which may correspond to detection of female-produced pheromone compounds by the male. This is in line with what previously was shown in derived moths that pheromone detecting neurons of sensilla trichoidea project into MGC and that these enlarged glomeruli are dimorphic and mostly present in males. Interestingly, in the butterfly *Bicyclus anynana* the MGC-like glomeruli seem to present only in female AL. In addition, the number of ORs found in the antennal transcriptome roughly correspond to the number of glomeruli's found in the antennal lobes of *R. nubila*, *E. semipurpurella* and *L. capitella*.

My work on olfaction in Trichoptera and primitive Lepidoptera has demonstrated that (1) receptors involved in detection of Type 0 and I pheromone compounds have possibly evolved independently from different plant volatile detecting ORs, (2) the functional studies of *L. capitella* PRs add functional support to the PR clade, and (3) some Lepidoptera specific chemosensory proteins are only present in *L. capitella* which use Type I pheromone for sex communication. This illustrates that the chemosensory gene families, at least at the level of antennal expression may be associated with different pheromone types. (4) Similarly, antennal morphology studies show a shift in major types of olfactory sensilla, from sensilla placodea in basal moths to sensilla trichoidea in derived moths.

Objective and research aim

Over the years, the sex pheromone communication system of moths (Lepidoptera) has become a model system for the understanding of evolution of sex pheromones and its counterpart, the pheromone receptors. However, the pheromone communication systems of basal Lepidoptera and the sister group of Lepidoptera, caddisflies (Trichoptera), have not yet been studied. Hence, the main objective of this thesis is to enhance our current knowledge on evolution of the sex pheromone communication system in moths by studying the basal species of Lepidoptera and their sister group Trichoptera. The components important for the function of the olfactory system such as olfactory sensilla, the primary olfactory processing centre of the antennal lobe, and families of chemosensory proteins and their function were studied. The main components of the work are to investigate the functional evolution of PRs and to identify chemosensory gene families, but I also document the diversity of antennal morphology, sensillar ultrastructure, and antennal lobe glomeruli architecture in some of the studied taxa.

I aim to, 1) explore the chemosensory gene families of *Rhyacophila nubila* (Rhyacophilidae: Trichoptera), *Eriocrania semipurpurella* (Eriocranidae; Lepidoptera) and *Lampronia capitella* (Prodoxidae: Lepidoptera) using next-generation sequencing technologies. 2) functionally characterize the pheromone receptors using HEK293 cells as the heterologous expression system. 3) study the antennal morphology and ultrastructure of the sensilla using scanning and transmission electron microscopy. 4) investigate the morphology of the primary olfactory centre, the antennal lobe and its glomeruli, using immunocytochemistry and confocal microscopy.

In addition to the results reported in the four papers (I-IV), I discuss the results from the immunocytochemistry studies of the antennal lobes and the glomeruli structures in the thesis introduction. These results are not yet complete and compiled in manuscript form.

Background

Organisms, including bacteria, fungi, plants, and animals, all have the ability to detect and respond to chemical signals. Insects have a remarkable olfactory system that allows them to detect large numbers of odor molecules with their specialized odorant receptors (ORs). Chemical ecology has become a fascinating field of study since it has been shown that pheromones and other semiochemicals play an important role in mate finding, host selection and predator avoidance. The field of chemical ecology was commenced when Butenandt et al. (1959) discovered the chemical attractant known as pheromone from the silk moth *Bombyx mori* (Bombycidae: Lepidoptera). Later developments in gas chromatography and mass spectrometry allowed chemical ecologists to identify the structure of pheromone components in many more species. Moth sex pheromones play an important role in successful reproduction (Löfstedt and Kozlov 1997; Wyatt 2014). In most cases females produce a species-specific blend and ratio of compounds (Roelofs and Jurenka 1996). The female produced pheromone compounds are perceived by males over a long distance using the antennae. Sex pheromones in moths are detected by pheromone receptors (PRs), a subfamily of ORs, located in the membranes of olfactory sensory neuron (OSN) dendrites.

Olfactory sensilla

The olfactory system of an adult insect consists of two main olfactory organs on the head; the antennae and the maxillary palps (Sato and Touhara 2009). The antenna is covered with numerous hairs called sensilla that hosts OSNs. However, the number of sensilla present on the antennae vary depending on the insect group and species, for instance psyllids and thrips have very few sensilla (Kristoffersen et al. 2006; Yuvaraj et al. 2013; De Facci et al. 2011) when compared to moths, flies and beetles (Hallberg 1982; Ebbinghaus et al. 1997; Anderson et al. 2000; Shanbhag et al. 2001; Ansebo et al. 2005). The airborne volatile signals are detected by the OSNs within olfactory sensilla. Insect antennae have different morphological types of olfactory sensilla; trichoidea, basiconica, chaetica, coeloconica, auricillica, placodea, styloconica and ampullacea, differentiated by their wall structure and shape (Keil 1999).

Insect olfaction

The sensilla house the dendrites of olfactory sensory neurons (OSNs) (Fig. 1), generally expressing one ligand-binding odorant receptor (OR) together with the ubiquitously expressed odorant receptor co-receptor (Orco). In moths, pheromone receptors are mostly found in the sensilla trichoidea (Hansson et al. 1995). In addition to ORs the insect OSNs express receptors from two other large and divergent gene families, namely ionotropic receptors (IRs) and gustatory receptors (GRs). In addition, sensory neuron membrane proteins (SNMPs) have been shown to be expressed in pheromone sensitive neurons and likely play an important role in pheromone detection (de Bruyne and Baker 2008; Kwon et al. 2007; Benton et al. 2007 and 2009; Touhara and Vosshall 2009; Rytz et al. 2013). The sensillum is filled with a protein-rich lymph, containing odorant binding proteins (OBPs) and odorant degrading enzymes (ODEs), which are involved in binding and breakdown of odorant molecules, respectively (Leal 2013) (Fig. 1). The cell membrane of the OSNs house the receptor proteins that bind odor ligands where the chemical signal is converted into an electrical signal that can be transmitted and processed by the nervous system including the antennal lobe and higher brain centres. The processed cues can provide fast and reliable information that induce innate or learned behaviors (de Bruyne and Baker 2008; Sato and Touhara 2009).

Chemosensory genes

Odorant and gustatory receptors

Unlike vertebrate ORs (Buck and Axel 1991), insect ORs are not homologous to seven transmembrane G protein-coupled receptors (GPCRs) (Clyne et al. 1999; Wistrand et al. 2006; Benton 2006). Perhaps the insect ORs have independently evolved as chemosensory proteins (Vosshall et al. 1999). The membrane topology of insect ORs is reverse to that of GPCRs, with an intracellular N-terminus and extracellular C-terminus (Benton 2006) (Fig. 1). The number of ORs found in different insect genomes varies depending on species, for example 62 ORs in the fly *Drosophila melanogaster* (Robertson et al. 2003), 79 ORs in the mosquito *Anopheles gambiae* (Hill et al. 2002), 170 ORs in the honeybee *Apis mellifera* (Robertson et al. 2006), 131 ORs in the mosquito *Aedes aegyptii,* (Kent et al. 2008), 341 ORs in the flour beetle *Tribolium castaneum* (Engsontia et al. 2008), 122 ORs in the gall midge *Mayetiola destructor* (Andersson et al. 2014),
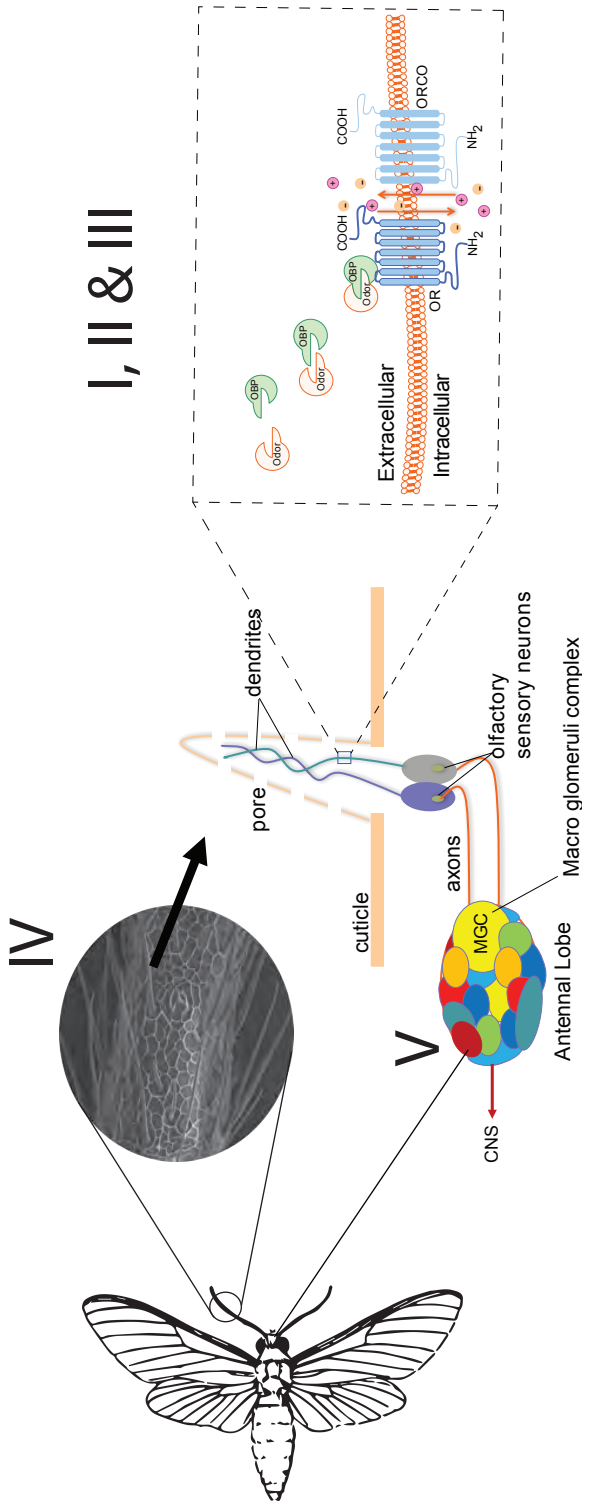
Figure 1. Schematic representation of the insect olfactory system and the different olfactory components studied in this thesis indicated with chapter numbers (I-IV). I-III) Identification and function of odorant receptor (OR), and olfactory receptor co-receptor (ORCO), the chapter III also deals with odorant binding protein (OBP), ionotropic receptors (IRs) and sensory membrane proteins (SNMPs). IV) Antennal morphology of *Rhyacophila nubila, Eriocrania semipurpurella, Lampronia capitella* and *Bicyclus anynana* were described. Inside image: morphology of the antenna from Lampronia capitella V) The primary olfactory center antennal lobe with the glomeruli (results are only discussed in the thesis introduction, not presented as a separate chapter).

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only 10 ORs reported so far in the human body louse (Kirkness et al. 2010) and the highest number of ~400 ORs in *Harpegnathos saltator* (Zhou et al., 2012). The number of ORs found in Lepidoptera differs based on species and sequencing (genome/transcriptome). For instance, genome assembly studies produced 66 ORs in *Bombyx mori* (International Silkworm Genome Consortium), 64 ORs in *Danaus plexippus* (Zhan et al. 2011), 95 ORs in *Plutella xylostella* (Engsontia et al. 2014) and 70 ORs in *Heliconius melpomene* (The Heliconius genome consortium 2012). On the other hand, antennal transcriptomes from a number of moth species have found different number of ORs, with 68 ORs in *Manduca sexta* (Grosse-Wilde et al. 2011), 58 ORs in *Cydia pomonella* (Walker et al. 2016), 47 ORs in *Spodoptera littoralis* (Poivet et al. 2013), and 70 ORs in *Epiphyas postvittana* (Corcoran et al. 2015). In moths, PRs have mainly been identified and functionally characterized from the families Noctuidae (Wang et al. 2011; Grosse-Wilde et al. 2007; Zhang and Löfstedt 2013), Bombycidae (Grosse-Wilde et al. 2006; Sakurai et al. 2004), Plutellidae (Sun et al. 2013), Saturniidae (Forstner et al. 2009), Crambidae (Wanner et al. 2010; Miura et al. 2010), Tortricidae (Steinwender et al. 2015; Corcoran et al. 2015) and Sphingidae (Wicher et al. 2017).

Both ORs and Gustatory receptors (GRs) belong to the same chemoreceptor superfamily. GRs are involved in contact chemoreception (taste) of sugars, salt, bitter tastants, and contact pheromones, and they are mainly located in the gustatory sensilla of insect proboscis, legs and wings (Robertson et al., 2003 and 2006; Vosshall and Stocker 2007). Some GRs are expressed in OSNs, where they notably function as carbon dioxide-detecting receptors (Kwon et al. 2007; Kent et al. 2008). GRs are found to be the most ancient chemosensory protein found in arthropods (present in basal invertebrate Placozoa) (Eyun et al. 2017).

Insect olfactory receptor gene families seem to evolve by birth-death evolution. In this process new genes arise through duplication events, whereas deletions and pseudogenization events represent the death of OR genes (Sánchez-Gracia et al. 2009; Ramdya and Benton 2010; Cande et al. 2013; Andersson et al. 2015; Benton 2015). This model is likely to apply also to PRs (Zhang and Löfstedt 2013). Pheromone receptors (PRs) in Lepidoptera are a subfamily of ORs that share sequence homology, and are usually more highly expressed in males. But it remains unknown how PR paralogues evolve in the duplication events under stabilizing selection (Zhang and Löfstedt 2013). The PRs form a highly conserved monophyletic clade that seems to evolve faster than ORs in general, particularly when compared to Orco (Carraher et al. 2012).

Odorant receptor co-receptor

The evolutionarily conserved odorant receptor co-receptor (Orco) forms heteromers of unknown stoichiometry with each ligand-binding OR (Vosshall and Hansson 2011). This receptor complex functions as ligand-gated ion channel (Sato et al. 2008; Wicher et al. 2008). In addition, Wicher et al (2008) suggested that metabotropic signaling might also occur. Orco is ubiquitously expressed in OSNs that express conventional ORs. Orco is also necessary for the ORs to localize and stabilize in the cell membrane of dendrites (Larsson et al. 2004; Benton et al. 2006; German et al. 2013) (Fig. 1). Unlike most other ORs, Orco is highly conserved among insects, sharing up to 94 % sequence identity among closely related species (Vosshall et al. 1999; Stengl and Funk 2013). Both Orco and conventional ligand-binding ORs are 7-transmembrane domain proteins. The predicted protein size of Orco is larger than the ORs, because of an insertion in the second intracellular loop.

Ionotropic receptors

Ionotropic receptors (IRs) are commonly expressed in coeloconic sensilla. IRs are related to ionotropic glutamate receptors (iGluRs) that belong to a highly conserved family of ligand-gated ion channels involved in synaptic functions in the vertebrate and invertebrate nervous system (Rytz et al. 2013). IRs have atypical binding domains compared to iGluRs, indicating that they have a different function (i.e. sensing the external environment) (Benton et al. 2009). IRs are more ancient than ORs as indicated by their presence throughout the protostome lineages which includes arthropods and nematodes among others (Croset et al. 2010). Insect IRs are divided into two subfamilies, 'antennal IRs' and 'divergent IRs'. The 'antennal IRs' in *Drosophila* are involved in salt, temperature and humidity sensing (Zhang et al. 2013; Enjin et al. 2016; Frank et al. 2017). The 'species-specific divergent IRs' are expressed in gustatory neurons involved in taste reception, at least in *Drosophila* (Croset et al. 2010). Unlike ORs, IRs are expressed in a combinatorial fashion in OSNs, and they are also tuned to different odorants, notably acids, aromatics, and nitrogen-containing compounds (Abuin et al. 2011).

Odorant binding proteins

Odorant binding proteins (OBPs) are small soluble and highly abundant proteins (typically 135–220 amino acids long) present in the sensillum lymph. They bind and solubilize hydrophobic odorants, e.g. pheromones (Sánchez-Gracia et al.

2009). OBPs act as mediators between the external environment and ORs in the dendrites of OSNs (Leal 2013). There are subfamilies of OBPs specialized for binding and carrying different classes of compounds, pheromone-binding proteins (PBPs), which are involved in pheromone detection (Vogt and Riddiford 1981; Grosse-Wilde et al. 2006), and general odorant binding proteins (GOBPs) involved in detecting general odorants (Liu et al., 2010). Both of these subfamilies appear to be conserved in most higher Lepidoptera (Ditrysia) (Vogt et al. 2015). Odorant degrading enzymes are involved in terminating the response of OSNs by enzymatic breakdown of odorants (Leal 2013).

Sensory neuron membrane proteins

Sensory neuron membrane proteins (SNMPs) are membrane bound proteins with two transmembrane domains, belonging to the CD36 protein family, which contains scavenging proteins. SNMPs are expressed in certain OR-expressing OSNs. The SNMP family has two members namely SNMP1 and SNMP2, and the exact copy number of each of the two SNMP members varies from 1-6 in different species (Nichols and Vogt 2008; Andersson et al. 2014). SNMP1 associates with pheromone-responding OSNs in *Drosophila* and in moths contribute to sensitivity in pheromone detection (Benton et al. 2007; de Bruyne and Baker 2008; Sanchez-Gracia et al. 2009; Leal 2013; Li et al. 2014; Pregitzer et al. 2014; Gomez-Diaz et al. 2016). In contrast, SNMPs are not necessary for the response of OR22a of *D. melanogaster* to fruit-related esters (Benton et al. 2007).

Antennal lobe and glomeruli

The axons of OSNs project into the primary olfactory centre in the brain, the antennal lobe (AL), that is comprised of spherical structures called glomeruli (neuropils) (Hansson and Anton 2000) (Fig. 1). The AL receives and processes the information from specific OSNs (Fig. 1). In derived moths, sex pheromone information has been shown to be processed by a group of dorsally located sexually dimorphic enlarged glomeruli called macro glomerular complex (MGC) (Hansson et al. 1991, 1992; Kanzaki et al. 2003; Vickers et al. 1998). The MGC consists of separate compartments, each compartment processes specific pheromone components in species using multicomponent pheromone blends (Hansson et al. 1991; Hansson 1997; Berg et al. 1998). Projection neurons (PNs) located between the AL and the higher brain centre receive and convey the processed information from the glomeruli to the higher processing centres. The

presence of MGC is sex-specific to male moths that have receptors specifically tuned to the pheromone component blends released by females. Counterparts of the MGC are also present in females, but those glomeruli are much smaller and tuned to odors that are relevant to female behavior (King et al. 2000). The remaining small glomeruli present in the AL are connected to specific OSNs on the antennae that are selectively tuned to a range of odorants that are important for both male and female behavior (Vickers et al. 1998).

Pheromone communication in moths and caddisflies

Pheromones are defined as chemical signals that are produced by an organism/individual and change the behavior or developmental events of another individual of the same species (Wyatt 2014). There are many kinds of pheromones used for various activities such as sex, aggregation, trail marking, and social status in a colony (Jurenka 2004). A sex pheromone can be a single compound or more commonly a blend of chemical compounds in a specific amount and ratio (Jurenka 2004), which is used to attract a conspecific mate for mating. In moths mostly females produce pheromone to attract males over long distance but in some species male pheromones act as display trait (Lassance and Löfstedt 2009; Wyatt 2014).

Moth sex pheromones are divided into four categories based on their site of production, chemical structure and biosynthetic features: Type 0, I, II and III. Type I pheromone compounds are the most commonly identified pheromones in Lepidoptera ($\sim$ 75% of moth species), which consist of C_{10}- C_{18} alcohols, acetates and aldehydes (Ando et al. 2004; Löfstedt et al. 2016) (Fig. 2). Type II pheromones are comprised of unbranched polyunsaturated hydrocarbons or the corresponding epoxy derivatives with longer (C_{17}-C_{25}) straight chains used by $\sim$ 15% of moth species (Ando et al. 2004; Löfstedt et al. 2016) (Fig. 2). The Type I and II sex pheromone-producing glands are located between the 8th and 9th abdominal segments (Ma and Ramaswamy 2003). Type III sex pheromones are branched (one or more methyl groups) long chain C_{17}-C_{23} saturated and unsaturated hydrocarbons, also functionalized hydrocarbons (Löfstedt et al. 2016). Type 0 pheromones are short-chain secondary alcohols and ketones and called so because they have been reported in the two oldest so-called nonditrysian lineages of Lepidoptera and their sister group Trichoptera (Fig. 2). The caddisfly, *Rhyacophila nubila* (Trichoptera: Rhyacophilidae) and the leaf miner moth *Eriocrania semipurpurella* (Eriocraniidae: Lepidoptera), have Type 0 pheromones, which are similar to general plant volatile compounds (Visser 1986; Löfstedt and Kozlov 1997).

Studies on pheromone biosynthesis have revealed that the genes that are involved in pheromone production evolve through gene duplication and structural mutation in the coding region (Wang et al. 2010b; Lassance et al. 2010). Considering the similarity between Type 0 and plant volatile compounds, it might also be possible that the Type 0 pheromone receptors might have evolved by gene duplication and structural mutation of ORs that are tuned to detect general plant volatiles. However, while PRs for Type I pheromones are well studied in more derived lepidopterans, pheromone receptors for Type 0 compounds in the basal Lepidoptera and in the Trichoptera have so far not been identified or functionally characterized. Furthermore, the PRs for Type I pheromones in Lepidoptera are evolutionarily related across species, with these receptors forming the specific lepidopteran "PR clade" in phylogenetic analyses. However, as no PRs for Type 0 pheromones have been identified, it is not known if these PRs are related to the PRs for Type I pheromones in Lepidoptera.
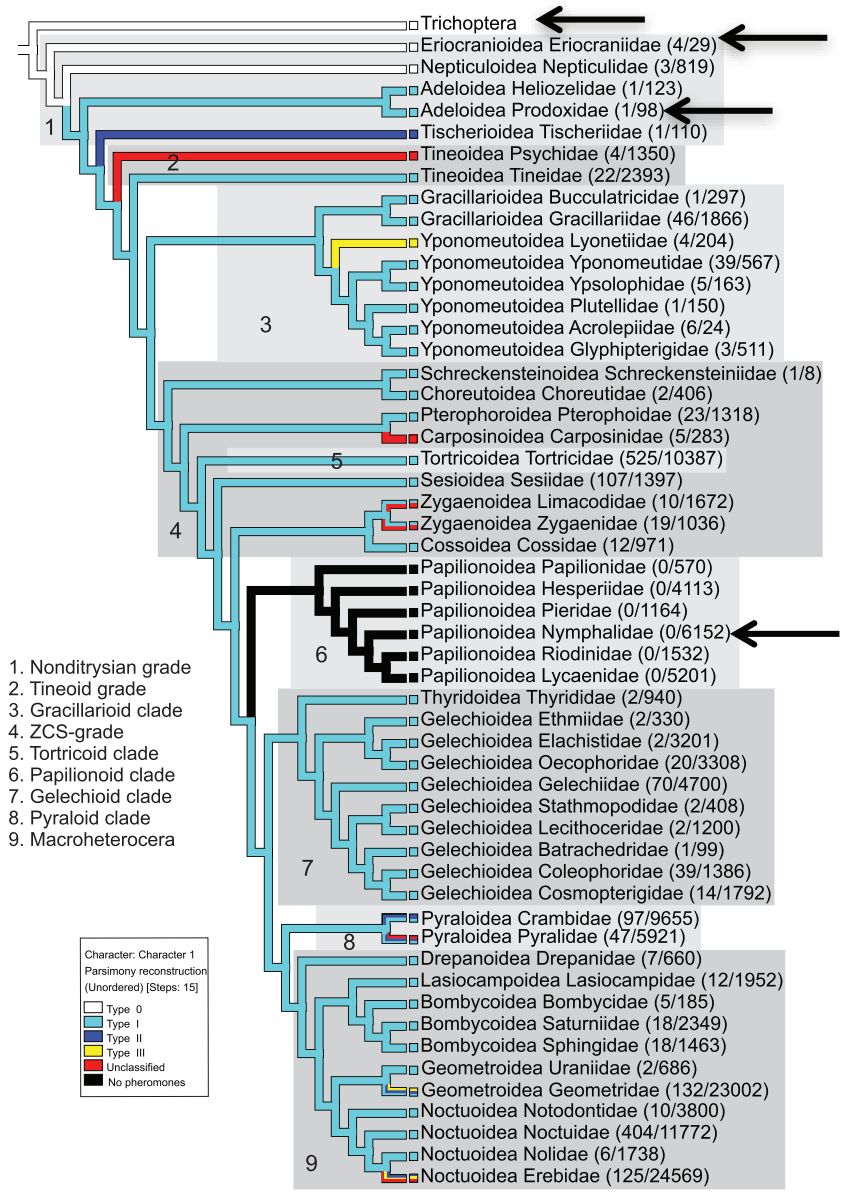
Figure 2. Pheromone types mapped on to a phylogenetic tree of Lepidoptera using the parsimony criterion. Numbers in parentheses after taxa indicate approximate number of pheromones and attractants reported, followed by the number of species in each taxon. Only taxa with reported pheromones or sex attractants are included in the tree. The black arrows indicate the family/order of the species studied in this thesis work (*R. nubila*, *E. semipurpurella*, *L. capitella* and *B. anynana*). The tree is adapted and modified from Löfstedt et al. 2016.

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Study organisms

Caddisfly, *Rhyacophila nubila*

Trichoptera are holometabolous insects with aquatic immature stages, and together with Lepidoptera form the superorder Amphiesmenoptera (Kjer et al. 2001, 2002). Thus, Trichoptera is the sister taxon of Lepidoptera. Trichoptera has ca. 10,000 described extant species grouped into 45 families (Morse 1997). Ancestors of caddisflies were possibly terrestrial, similar to scorpion-flies and lived in moist places (Nilsson 2006). Trichoptera and basal Lepidoptera have similar types of pheromone components (Type 0) and the same type of pheromone-producing structures (Löfstedt and Kozlov 1997; Löfstedt et al. 1994, 2008). Solem (1985) reported that the extracts of the IVth abdominal sternite from females of *R. nubila* attract males. Later it was shown that the *R. nubila* (Rhyacophilidae; Trichoptera) (Fig. 3A) pheromone was produced by exocrine glands located in the IVth and V[th] abdominal sternites of females, and male antennae were electrophysiologically activated by the pheromone (Löfstedt et al. 1994). Female *R. nubila* produces nearly equal amounts of heptan-2-ol, heptan-2-one, and nonan-2-one, but also a smaller amount of nonan-2-ol (Löfstedt et al. 1994). Male *R. nubila* produces acetophenone, hexanoic acid, and octanoic acid, but their role in pheromone communication has not been studied (Ansteeg and Dettner 1991; Löfstedt et al. 1994). Single sensillum recordings (SSR) on *R. nubila* males and females showed responses to heptan-2-one, (*R*)-heptan-2-ol, (*S*)-heptan-2-ol, nonan-2-one, (*R*)-nonan-2-ol, and (*S*)-nonan-2-ol, where four OSN types are involved in the reception (Larsson and Hansson 1998).

Birch leafminer moth, *Eriocrania semipurpurella*

The leaf minermoth, *Eriocrania semipurpurella*, belongs to the non-ditrysian family Eriocraniidae. Among the Lepidoptera, Eriocraniidae is one of the more basal moth families. The presence of *E. semipurpurella* has been reported in birch forests in North America, Europe and Japan (Bylund and Tenow 1994; Imada et al. 2011) where the females lay eggs in flower buds and the larvae feed on the leaves of the trees (Bylund and Tenow 1994). After four larval instars, *E. semipurpurella* overwinters as a cocoon in the soil with the day-active adults

emerging during early spring depending on the geographic location (Bylund and Tenow 1994). Eriocraniidae uses the Type 0 pheromone similar to Trichoptera (Zhu et al. 1995; Kozlov et al. 1996), whereas most of the more derived moths use Type I and II pheromone compounds (Ando et al. 2004; Löfstedt et al. 2016). *E. semipurpurella* is the most common and widespread among the *Eriocrania* species that feed on birch, most *Eriocrania* species fed on trees of Betulaceae family (Fig. 3B). The exocrine glands are located on the fifth abdominal segment of female *E. semipurpurella* and produce the sex pheromone components (2*S*,6*Z*)-6-nonen-2-ol and (2*R*,6*Z*)-6-nonen-2-ol (Kozlov et al. 1996). These pheromone compounds are attractive to males but not to females (Larsson et al. 2003). In addition, the female gland also contains presumed precursors, (*Z*)-6-nonen-2-one and nonan-2-one that are most abundant compounds in the pheromone gland extracts of *E. semipurpurella*. When these ketones were included in a pheromone blend in a field trapping experiment, the ketones acted as antagonists for both *E. semipurpurella* and *E. sangii* (Kozlov et al. 1996). In contrast, low concentration of nonan-2-one, but not of (*Z*)-6-nonen-2-one, synergized pheromone attraction, suggesting it might in fact be a pheromone synergist at low concentrations (Larsson et al. 2002). SSR study showed that male *E. semipurpurella* have five OSN types which respond to pheromones of *E. semipurpurella* and structurally similar pheromone compounds from closely related species (Larsson et al. 2002).

The currant shoot borer moth, *Lampronia capitella*

The currant shoot borer, *Lampronia capitella* (Lepidoptera; Prodoxidae) is a monotrysian moth (females have a single genital opening for mating and egg laying), and is a serious pest on red and black currants (Fig. 3C). Young *L. capitella* larvae feed on the fruits of currant or gooseberry (*Ribes* spp.). The larvae overwinter near roots of the host plants and the larvae feed on the young buds of the currant plants during spring. Based on currently available pheromone data within Lepidoptera, Adeloidea containing the families Prodoxidae and Heliozelidae is the first branch in the phylogeny with Type I pheromone compounds (Löfstedt et al. 2016, Fig. 2). The females of *L. capitella* produce (Z9,Z11)-tetradecadienol (Z9,Z11-14:OH), (Z9,Z11)-tetradecadienal (Z9,Z11-14:Ald), and (Z9,Z11)-tetradecadienyl acetate (Z9,Z11-14:OAc) from their pheromone gland which is located on the extended terminal abdominal segment. GC-EAD studies on male *L. capitella* showed that the pheromone compounds are antennally active (Löfstedt et al. 2004) and field trapping experiments showed that all three pheromone compounds of the pheromone blend are important for the maximum attraction of males. Z9,Z11-14:OH is the main pheromone compound

and necessary for attraction, whereas absence of either Z9,Z11-14:Ald or Z9,Z11-14:OAc resulted in reduced trap catch (Löfstedt et al., 2004).
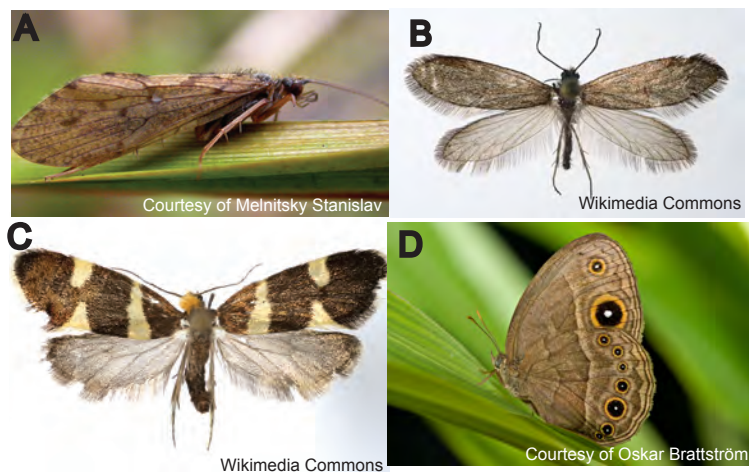

Figure 3. Images of study organisms. A. *Rhyacophila nubila* (Trichoptera: Rhyacophilidae), B. *Eriocrania semipurpurella* (Lepidoptera: Eriocraniidae), C. *Lampronia capitella* (Lepidoptera: Prodoxidae). D. *Bicyclus anaynana* (Nymphalidae: Lepidoptera).

The squinting bush brown, *Bicyclus anynana*

Butterflies are group of insects belong to a monophyletic clade that nested within Lepidoptera. Butterflies are evolved moths with different ecology. The squinting bush brown, *Bicyclus anynana,* belongs to the family Nymphalidae, the most diverse group of butterflies (Wahlberg et al. 2003) (Fig. 3D). *B. anynana* has been used as a model to study speciation and the evolution of sex pheromones among closely related races and species. *B. anynana* uses male sex pheromones for short-range courtship behavior. Interestingly, some of the pheromone compounds of *B. anynana* are structurally identical to many known female moth sex pheromone components. Male *B. anynana* produces Type I pheromone compounds on their wings which consist of (*Z*)-9-tetradecenol (Z9-14:OH), hexadecanal (16:Ald) and 6,10,14-trimethylpentadecan-2-ol (6,10,14-trime-15-2-ol) (Nieberding et al. 2008, 2012). The electrophysiological (GC-EAD) studies shows that 16:Ald, Z9-14:OH and 6,10,14-trime-15-2-ol are antennaly active (Nieberding et al. 2008). However, no behavioral evidence has been shown for the activity of male pheromone compounds in this species.

General methodology

Transcriptome analysis

Transcriptome sequencing is a technique used to identify and quantify the presence of RNA expressed in a specific tissue of an organism during a particular time of their life-cycle. The chemosensory genes are transcribed and present as messenger RNA (mRNA) in the antennae. Transcriptome sequencing was used to identify the chemosensory genes present in the antennae. The total RNA was extracted from the antennae followed by construction of a cDNA library (Andersson et al. 2014; Corcoran et al. 2015; Yuvaraj et al. 2017) (Fig. 5). The cDNA was then sequenced using an Illumina Hiseq 2000 platform at Beijing genomics institute (BGI, Hong Kong Co., Ltd.). When there is no reference genome for a transcriptome data, the *de novo* sequence assembly is created by assembling the short cDNA sequence reads together to obtain longer reads (unigenes). Sequence reads were assembled *de novo* using the Trinity assembler (Version20121005, Grabherr et al. 2011). Functional annotation of unigenes was performed by blasting against a pooled database of nonredundant (nr) proteins at NCBI (National centre for biotechnology information). A detailed protocol used for transcriptome analysis is given in the methods section of Chapter III. The methods for antennal transcriptome sequencing and data analysis of chemosensory genes are illustrated in Fig. 4.
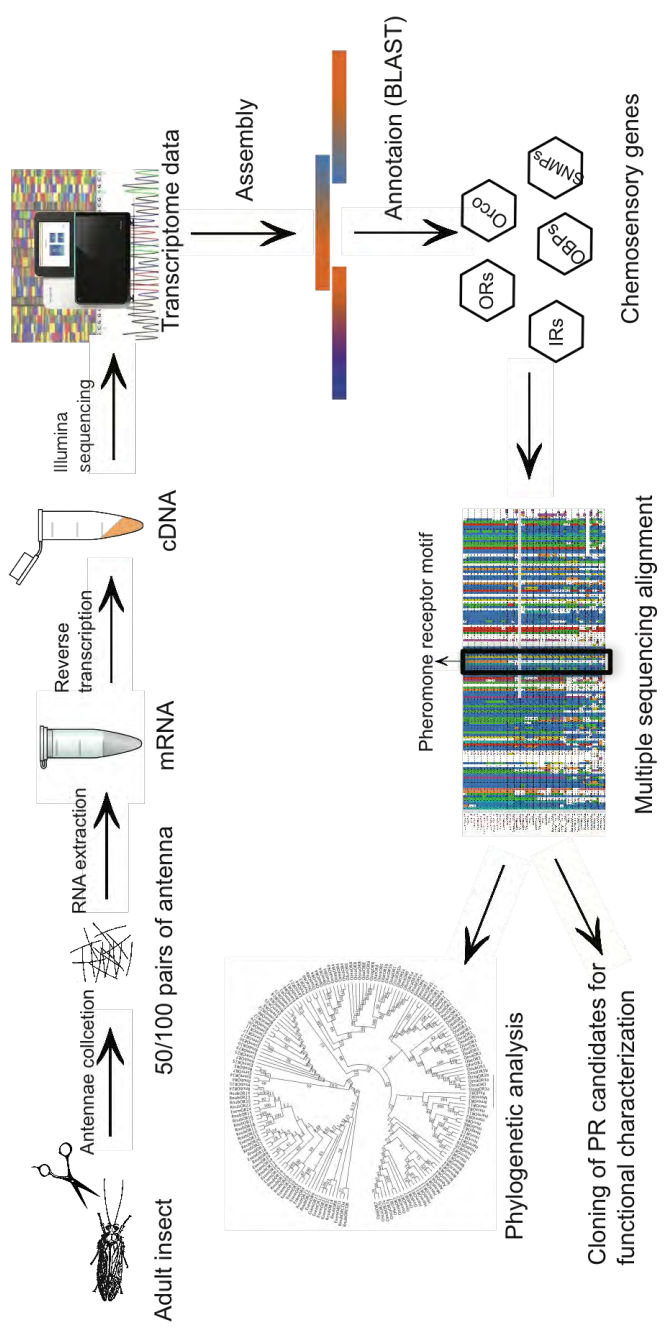
Figure 4. Schematic overview of the workflow from antennae collection to identification of chemosensory genes for phylogenetic and functional studies.

Functional characterization using HEK293 cells

Full length OR genes identified from a transcriptome can be used for functional characterization using various *in vivo* and *in vitro* methods. The *in vivo* approach is generated by adding the gene of interest to a *Drosophila* neuron that lacks an endogenous odorant receptor (Ha and Smith 2006; Hallem et al. 2004). This method has been widely used to study ORs from Diptera and some moths (Syed et al. 2006; Ueira-Vieira et al. 2014; de Fouchier et al. 2017). When instead using *in vitro* methods, the OR genes have mostly been characterized in Sf9 cells and *Xenopus* oocytes (Kiely et al. 2007; Sakurai et al. 2004). Both *in vivo* and *in vitro* systems are providing platforms to study ligand detection (sensitivity and selectivity) at the receptor level. Both of these methods have their advantages and disadvantages, in general none is suitable for high-throughput screening. Another significant disadvantage is that the gene expression cannot be controlled in oocytes and Sf9 cells, so the cells can have different numbers of the receptors in their membrane. Recently a novel *in vitro* method using human embryonic kidney cells (HEK293) was developed for the functional characterization of insect odorant receptors (Corcoran et al. 2014). Briefly, wild type HEK293 cells are transfected with inducible receptor constructs allowing for control of gene expression. The plasmids expressing the Orco and OR are transfected into the HEK cells expressing a Transcriptional Repressor (TREx). Approximately after 12 weeks, stable cell lines expressing the gene of interest are obtained (Fig. 5). Using a fluorescent spectrophotometer, changes in ligand-induced OR activation can be monitored using Ca^{2+}-sensitive dye (Fig. 5). Detailed descriptions of how stable cell lines are generated and of the fluorescent calcium assay method are presented in Chapters I and II (see also Corcoran et al. 2014).
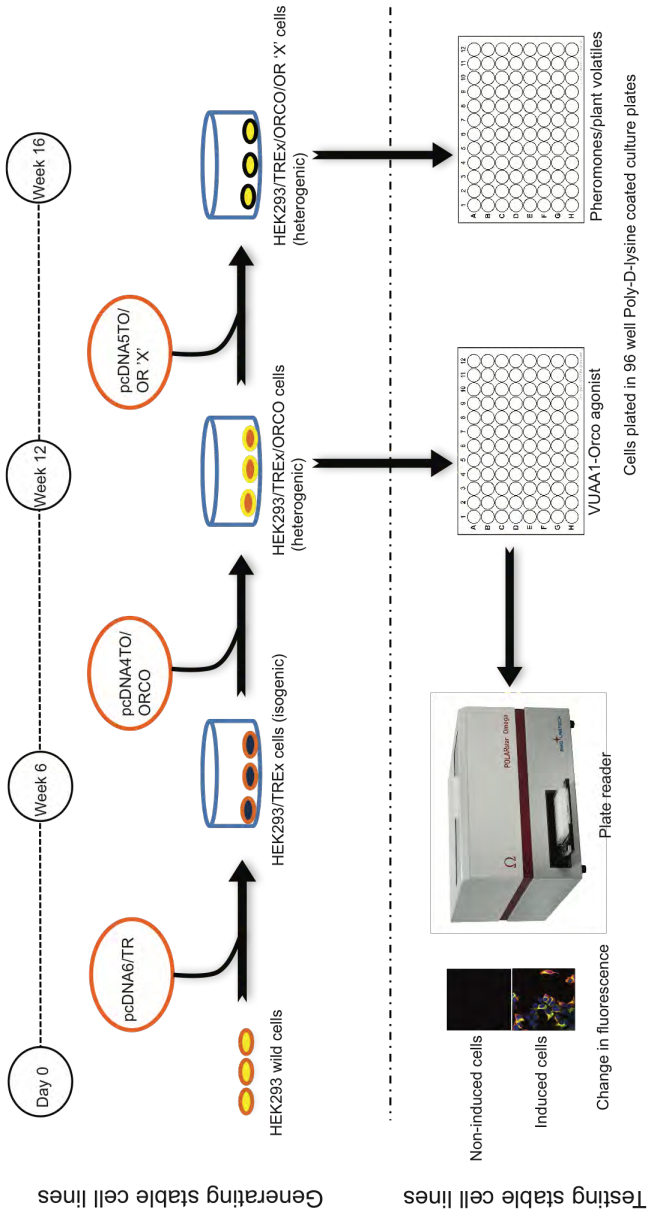
Figure 5. Schematic illustration of generating stable heterogenic cell lines expressing HEK293/TRex/ORCO/OR'X'. The dashed lines with circles indicates appoximate amount of time required to make the respective cell lines. The testing phase includes loading of cells in 96 well plates with fluorescent dye and measurement of change in fluorescence intensity using a plate reader. Adapted inside Images: Plate reader (Biocompare), induced and non-induced cells (Corcoran et al. 2014).

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Microscopy studies

Scanning and transmission electron microscopy imaging techniques were used to examine the sensillar equipment of the antennae and to characterize the morphology and ultrastructure of the olfactory sensilla (detailed description in paper IV; De Facci et al. 2011). Briefly, the samples for scanning electron microscopy (SEM) imaging were fixed and dehydrated in fixative solution and a graded ethanol series, respectively, followed by critical point drying and sputter coated with gold. Transmission electron microscopy (TEM) samples were also fixed and dehydrated before sliced into ultra-thin sections using a diamond knife. The SEM preparations were imaged using a SEM Hitachu SU3500 at 5 kV. The TEM samples were examined using JEOL JEM 1400 plus transmission electron microscope.

Immunocytochemistry of whole mount preparations

The whole-mount staining protocol was followed as described in Stöckl and Heinze (2015). The brain size of *R. nubila*, *E. semipurpurella* and *L. capitella* was smaller than the hawkmoths used in Stöckl and Heinze (2015) so the incubation and washing times were shortened. But, *B. anynana* brain size was similar to that of most derived moths, therefore, standard incubation and washing times were used as described in Stöckl and Heinze (2015), which is indicated in parenthesis. Briefly, the brains were dissected and fixed in ZnFA fixative (18.4mM Zn Cl_2, 135mM NaCl, 35mM sucrose, and 1% paraformaldehyde, Ott 2008) overnight followed by 8 x 10min (15 min) wash with Hepes Buffered Saline [150 mM of NaCl, 5 mM KCl, 5mM $CaCl_2$, 25mM sucrose, 10mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid)], (Stöckl and Heinze 2015). The brain samples were then incubated for 20min (60 min) with 20:80 DMSO/methanol to increase the tissue permeability followed by 3 x 7min (10 min) wash with Tris-HCl (0.1 M Tris-buffered saline). Then the brains were pre-incubated with 5 % NGS diluted in 0.01M PBT (phosphate buffered saline (PBS) with 0.3% TritonX-100) for 4 h at room temperature (overnight at 4°C). After that, the brains were stained with primary antibody anti-synapsin (1:25 in in PBT with 1% NGS) for 2 days (4 days) at 4°C and washed for 8 x 10 min (15 min) with PBT. The secondary antibody staining was performed with GAM-Cy5 (1:300 in PBT with 1% NGS) at 4°C for 1 day (2 days) followed by washing for 6 x 10min (15 min) in PBT and 2 x 10min (15 min) in 0.01M PBS. The brain samples were then dehydrated in an ethanol series of increasing concentrations (50, 70, 90, 95

and 2 x 100%; 10 min (15 min) each) and cleared in methyl salicylate, first with 1:1 of ethanol and methyl salicylate for 10 min and then with pure methyl salicylate for 30 min. Brains were mounted in Permount (Electron Microscopy Science, Hartfield, PA, USA) between two #1.5 coverslips with plastic spacers (5 reinforcement rings (8 for *B. anynana*) at ca. 80 μm each from Zweckform No.3510, Germany) to avoid squeezing of the brains.

Confocal imaging and 3D reconstruction

The whole mount preparations were imaged using 633nm HeNe laser at a confocal microscope (LSM 510 Meta, Zeiss, Jena, Germany) with a 25x objective (LD LCI Plan Apochromat 25x/0.8 Imm Corr DIC, Zeiss) and a frame size of 1024x1024 voxels with optical sections every 1 μm. This resulted in a voxel size of 0.4972x0.4972x1.0387 μm. The detector range was set to 646nm-753nm, pinhole to 1 airy unit. Reconstruction of the confocal image stacks was performed using Amira segmentation editor (Version 5.5.3, 3D visualization and analysis software from FEI part of Thermo Fisher Scientific). The volume rendering of the antennal lobe was performed using the "voltex" tool in Amira.

Results and discussion

Evolution of chemosensory gene families (Paper III)

A transcriptomic approach was used to identify the chemosensory genes from the antennae of the three study species. Bioinformatics analysis of antennal transcriptomes revealed different numbers of OR transcripts, 52 in *L. capitella*, and 37 in both *R. nubila* and *E. semipurpurella*. The numbers of ORs found in *R. nubila* and *E. semipurpurella* were lower than the numbers found in pervious transcriptomic and genomic studies of moths (Zhan et al. 2011; Engsontia et al. 2014; Corcoran et al. 2015; Walker et al. 2016). However, the actual numbers of ORs are likely to be larger in the genomes than what was found in the antennal transcriptomes. In general, each OSN express one OR and all OSNs expressing the same OR project to the same glomeruli (reviewed in Andersson et al. 2015). However, there are few exceptions when OSNs expressing multiple ORs (Dobritsa et al. 2003; Koutroumpa et al. 2014; Karner et al. 2015). Additionally, in *Drosophila*, OSNs expressing IRs also project to individual glomeruli in the AL (Silbering et al. 2011). Several studies have showed correlation between the number of ORs and olfactory glomeruli in the AL (Vosshall et al. 2000; Vosshall and Stocker 2007; Grosse-Wilde et al. 2011). Our preliminary AL re-construction results indicate that *R. nubila* and *E. semipupurella* antennal lobes have lower numbers of glomeruli than *L. capitella,* which roughly correlates with the differences in the number of ORs found from the antennal transcriptomes (see section: Antennal lobe organisation).

The number of IRs ranged from 17 to 19, and 30, 23, and 29 OBPs were found in *R. nubila*, *E. semipurpurella* and *L. capitella*, respectively. IR transcripts for conserved IRs (IR1, IR8a, IR21a, IR25a, IR40a, IR41a, IR60a, IR68a, IR76b, IR87a, IR93a, and members of the IR75 group) were found in the antennal transcriptomes, however, some of the IR orthologs were not found in all of the three species. For example, IR64a, IR75d and IR75p orthologs were not found in *R. nubila* and IR7d was not identified in *E. semipurpurella*. Different subgroups of OBPs follow interesting evolutionary patterns. No GOBPs/PBPs were found in *R. nubila* and no PBPs were found in *E. semipurpurella,* but two PBPs were found in *L. capitella.* The functional characterization study identified two PRs from *L. capitella* responding to Type I pheromone compounds (Chapter II). The presence of PBPs and PRs within the PR-clade of Lepidoptera (Koenig et al. 2015) found

only in *L. capitella* but not in *R. nubila* and *E. semipurpurella* suggests that these PRs and the PBPs may have evolved in parallel with the switch to Type I pheromone compounds (Fig. 6), at least when considering antennal expression. Genome analysis of these species would further improve our understanding of the evolution of these chemosensory gene families in Lepidoptera (i.e. the genes might be present in the genome, but not expressed in the antennae). Two SNMP orthologs (SNMP1 and SNMP2) were found in all three species. In *L. capitella* we found two orthologs of SNMP1 which is not entirely surprising because several insect species have shown to contain multiple members of SNMP1 (Nichols and Vogt, 2008; Andersson et al. 2013; Andersson et al. 2014). The LcapSNMP1a shares high sequence similarity to SNMP1 orthologs in derived moths. However, LcapSNMP1b is relatively divergent to the SNMP1 in the basal Lepidoptera and Trichoptera (Paper II). In *Drosophila* SNMP1 has been shown to be 1) necessary for cVA response, 2) instead affects response onset and offset, and 3) improves sensitivity (Benton et al. 2007; Li et al. 2014; Gomez-Diaz et al. 2016). Also in some derived moth species, SNMP1 has been shown to affect the sensitivity of the pheromone response (Li et al. 2014; Pregitzer et al. 2014). Hence, the SNMP1 in Trichoptera and basal Lepidoptera might be important for pheromone detection, whereas the role of SNMP2 is still unknown. Our study provides the first and only so far data set for the chemosensory gene families of the basal Lepidoptera, as well as of out-group Trichoptera.
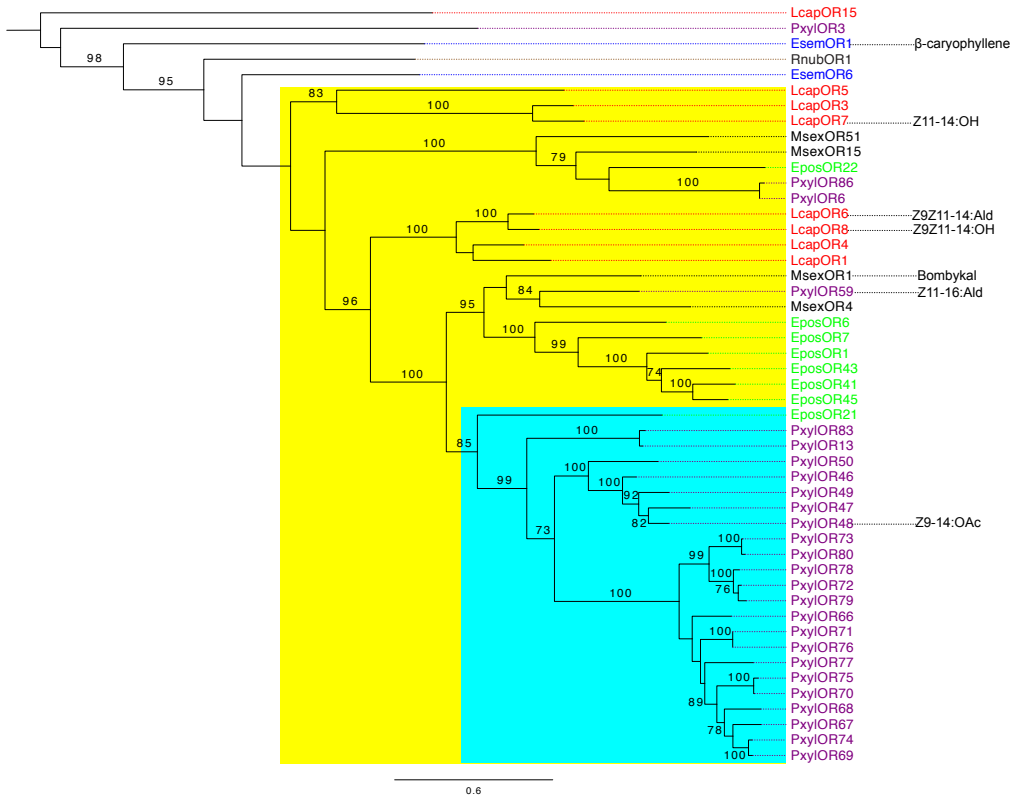
Figure 6. Maximum-likelihood phylogram based on the protein sequences of odorant receptors (ORs) from *Rhyacophila nubila* (black), *Eriocrania semipurpurella* (blue), *Lampronia capitella* (red), *Epiphyas postvittana* (green), *Manduca sexta* (black), and *Plutella xylostella* (purple). Only the monophyletic clade containing pheromone receptors (PRs) and closely grouped ORs are shown. The PR clade is marked in yellow, the ORs included in the recently expanded PR clade in cyan. The best agonist of each receptor is indicated based on results from the present and pervious functional characterization studies (Sun et al. 2013; Wicher et al. 2017; Yuvaraj et al. 2017). Bootstrap support values shown if >70.

Evolution of sex pheromone receptors (Paper I and II)

The functional studies were aimed to characterize odor response profiles of ORs involved in pheromone detection of *E. semipurpurella* and *L. capitella*. Using HEK293 heterologous expression system, I functionally characterized three receptors from *E. semipurpurella*: EsemOR1 that responded to a plant volatile and

EsemOR3 and 5 both responded primarily to Type 0 pheromones and to a smaller extend to plant volatiles. Three ORs from *L. capitella* (OR6, 7 and 8) that responded to Type I pheromone compounds. EsemOR3 and 5 strongly responded to the pheromone compounds and antagonist of *E. semipurpurella,* (2*S*,6*Z*)-6-nonen-2-ol and (*Z*)-6-nonen-2-one, respectively (Fig. 7A and B). In addition, EsemOR3 and 5 also responded weakly to the structurally similar common plant volatile compounds (Fig. 7A and B;Yuvaraj et al. 2017). Our phylogenetic analysis together with the functional characterization of PRs shows that the receptors for Type 0 and I pheromone compounds are not phylogenetically related, rather, they group at different positions in the OR phylogenetic tree. However, RnubOR1 from *R. nubila* (Chapter III), and two receptors from *E. semipurpurella* (OR1 and 6) group at the base of the lepidopteran PR clade that contains receptors for Type I and II pheromone compounds (Koenig et al. 2015; Yuvaraj et al. 2017) (Fig. 6). One of them, EsemOR1, specifically responded to a plant volatile compound, β-caryophyllene, among the large panel of odor ligands tested (Fig. 7C; Yuvaraj et al. 2017). In these studies, (Chapter I and II), I have reported the first functionally characterized pheromone receptors for Type 0 pheromones and also the first pheromone receptor for Type I pheromones from a non-ditrysian moth. Our results showed that PRs for Type 0 most likely have evolved from ORs tuned to detect structurally similar plant volatiles. In addition, the grouping of the plant volatile receptor EsemOR1 at the base of the PR clade suggesting that PRs for Type I pheromone compounds may have also evolved independently from ORs tuned to detect plant volatile compounds. Thus, Type 0 and I PRs are not closely related and they have evolved from different ancestral proteins that may have been tuned to detect plant volatiles.
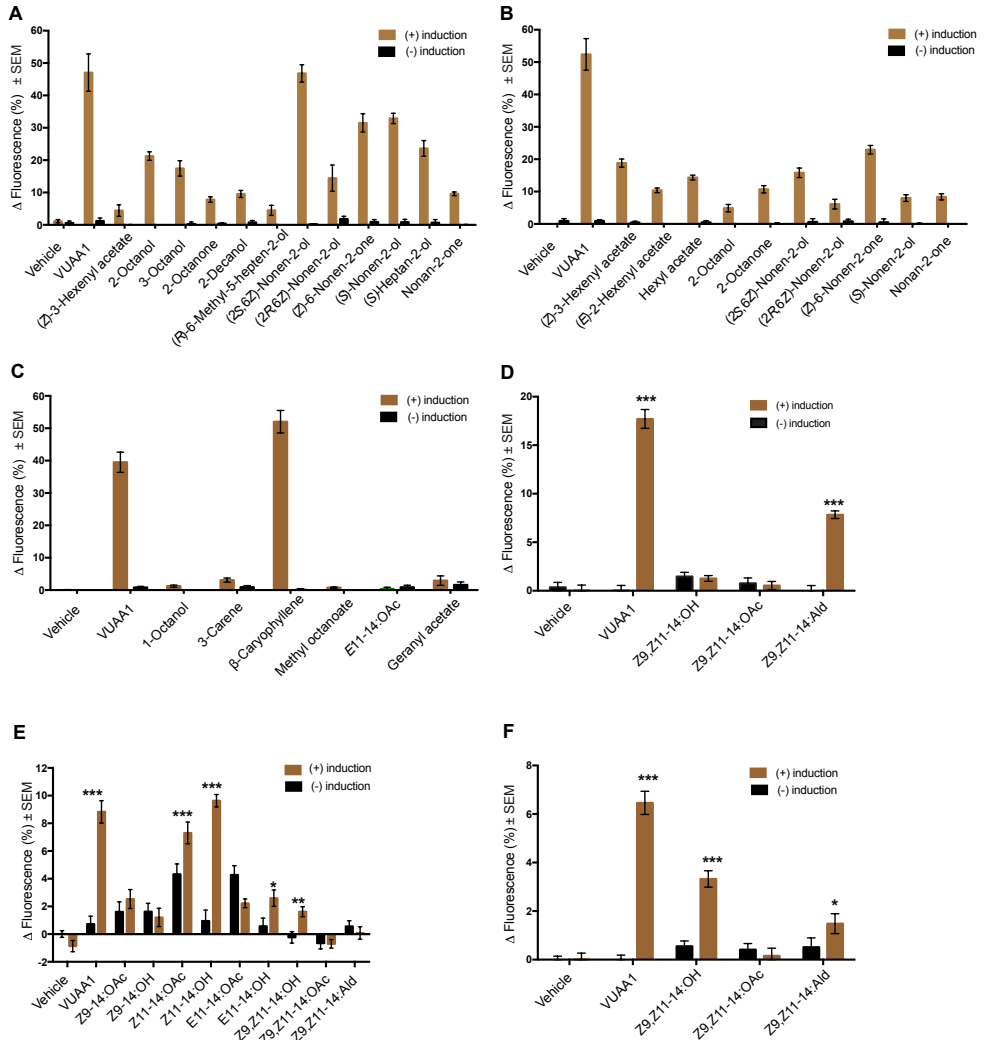
Figure 7. Response of HEK293 celles transfected with Orco and ORs of *Eriocrania semipurpurella* and *Lampronia capitella* to vehicle control (0.5%DMSO), the Orco agonist VUAA1 (50 μM) and various pheromone comopunds and plant voaltiels. (A) EsemOrco/OR3 (B) EsemOrco/OR5 (C) EsemOrco/OR1 (D) LcapOrco/OR6 (E) LcapOrco/OR7 and (F) LcapOrco/OR8. Plotted values are the mean response of three biological replicates (±SEM) from induced cells (green bars) and non-induced cells (black bars).

Selection of candidate PRs for functional testing was particularly focused on the presence of PR motifs, grouping with plant volatile responding ORs and higher expression level (Yuvaraj et al. 2017; Chapter II) (Fig. 8). PR-motifs are repeated patterns of amino acids that are conserved across moth species (Fig. 8). These motifs are serving as a useful site for designing degenerate primers that helps to amplify new PRs from the cDNA for species which does not have genome or transcriptome sequenced. The alignment of previously identified moth PRs sequences shows a conserved C- terminal that consists of conserved motifs (Fig. 8; details, Zhang and Löfstedt 2015). Site-directed mutagenic studies on the residue 'E' in the PR motifs with signature sequence L-(L/M)-(L/V)-(E/Q)-C-(S/T/A) or P-W-(E/Q/D) in *Bombyx mori* OR1 (BmorOR1) showed effects on spontaneous and odor-evoked action potentials on OR-Orco complex, indicating the importance of these residues (Nakagawa et al. 2012). The amino acid residues in the PR motifs are highly conserved in the ORs of the PR clade than the ORs that are grouping on the base of the PR clade. For instance, RnubOR1, EsemOR1and 6 fall on the base of the PR clade and have less residue identity. On the other hand, the LcapORs in the PR clade have more of these residues identical to those in derived moths. Surprisingly, the PRs (EsemOR3 and 5) for type 0 pheromones that group in different positions of the phylogeny also partially contain these residues. The role of these residues in the PR motifs is still unclear. Perhaps changes in these motifs may alter protein structure which can affect OR-Orco, OR-SNMP interactions or ion channel formation. The general conservation of ORs at the C-terminal suggests that it plays a general role such as forming an ion channel. However, mechanism of function of the residues in the PR motifs remains to be investigated.
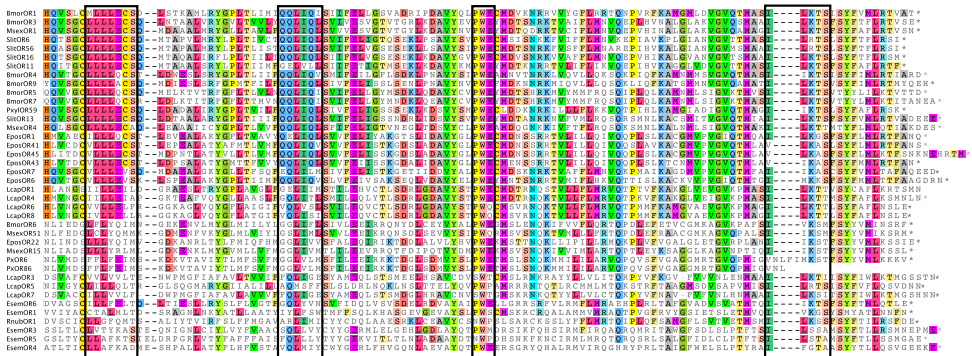
Figure 8. Multiple sequence alignment of C-terminal regions of the odorant receptors (ORs) from the "PR clade" of *Bombyx mori* (Bmor), *Epiphyas postvittana* (Epos), *Manduca sexta* (Msex), *Plutella xylostella* (Pxyl), *Spodoptera littoralis* (Slit), and PR candidates from *Eriocrania semipurpurella* (Esem) and *Lampronia capitella* (Lcap). Colours indicate identical amino acids by amino acid type and conserved motifs are highlighted with black rectangles.

The two of the three characterized ORs from *L. capitella* (OR6 and 8) grouped next to the already defined PR clade (Fig. 8). The monophyletic clade of *L. capitella* ORs (1,4,6 and 8) separates the 'sex-biased OR clade' (suggested as an extended PR clade in Koenig et al. 2015) from the 'classical' PR clade. Another *L. capitella*-specific clade basal to the 'sex-biased OR clade' contain LcapOR3, 5 and 7, among those LcapOR7 responded to typical Type I pheromone compounds (Chapter II). The functional evidence for LcapOR6, 7 and 8 suggest that the clades that contain these functionally characterized ORs can be regarded as PRs and thus be included in the PR clade (Fig. 8). Hence, the PR clade includes the two *L. capitella*-specific clades, the 'sex-biased OR clade' and the recently expanded PR clade (Koenig et al. 2015) (Chapter II and III). However, at this point, these receptors in the sex-biased receptor clade do not have any functional evidence, but based on their current phylogenetic position it is likely that they do respond to Type I pheromones. However, future functional support for these receptors in the 'sex-biased OR clade' will strengthen our conclusion. Thus, more functional characterization studies of OR from both basal and derived moths, including the ORs from the basal lineages of the PR clade, will help us to elucidate the origin of the PR clade.

In total, I functionally tested 23 ORs from Rnub (11 ORs), Esem (5 ORs) and Lcap (7 ORs). Six of them responded to the compounds included in the odor panel. The expression of OR and Orco proteins in the cell lines were confirmed using western blot for *E. semipurpurella* and *L. capitella* but not on *R. nubila* cell lines. The lack of response of most ORs could be due to that 1) ORs are expressed insufficiently or not translated in sufficient quantities, 2) they might not be incorporate properly in the cell membrane, or 3) the odor panel does not contain the specific ligand of the receptor. The lack of response is not surprising considering previous functional studies using heterologous expression systems where not all the receptors respond to any tested ligands (Hallem and Carlson 2006; Carey et al. 2010; Wang et al. 2010a; Andersson et al. 2016; Yuvaraj et al. 2017).

Diversity of olfactory sensilla (Paper IV)

The SEM and TEM studies identified six different morphological types of olfactory sensilla on the antennae of *R. nubila*, *E. semipurpurella* and *L. capitella* based on morphology and ultrastructure images (Table 1; Paper IV) (Fig. 9). The six sensilla types are: mushroom-like pseudoplacoid, forked pseudoplacoid, auricillica, trichoidea, basiconica and coeloconica (Table 1; Paper IV). No clear sexual dimorphism between males and females were observed, except in *L.*

capitella, where males seem to have more long sensilla trichoidea than females. The antennae of Trichoptera have different forms of sensilla placodea, mushroom-like, forked, horn-like, stellate, coronal, bilobed and dentate pseudoplacoid (Ivanov and Melnitsky 2016; Table 1; Paper IV) and sensilla auricillica in Lepidoptera resembles that of mushroom-like placoid in Trichoptera (Anderson et al. 2000; Larsson et al. 2002; Chapter IV). The placoid type of sensilla are present in higher numbers than other sensilla types in *R. nubila* and *E. semipurpurella* (Fig. 9). The antennae of *L. capitella* and *B. anynana* contain higher numbers of sensilla trichoidea and intermediate numbers of sensilla basiconica (Fig. 9). On the other hand, *L. capitella* and *B. anynana* lack the placoid type of sensilla, likewise both *R. nubila* and *E. semipurpurella* lack sensilla basiconica. However, the antennae of some derived moths contain sensilla auricillica (Anderson et al. 2000). I observed a major shift from the sensilla placodea to sensilla auricillica and then to sensilla trichoidea which may correlate with the detection of certain types of behaviorally important volatile cues such as different types of pheromones. For instance, electrophysiological studies showed that sensilla auricillica is involved in detection of Type 0 sex pheromone compounds in *E. semipurpurella* (Larsson et al. 2003). In many moths sensilla trichoidea have been shown to be involved in detection of Type I and II pheromone compounds (Mochizuki et al. 1992; Hansson et al. 1995; Ebbinghaus et al. 1997). However, morphological data from more basal moth species and electrophysiological studies from different sensillum types may help to clarify the specific function of the different sensilla types.

Table 1.

Summary of sensilla types found in *Rhyacophila nubila*, *Eriocrania semipurpurella*, *Lampronia capitella* and *Bicyclus anynana*.

Sensilla type Species	Mushroom-like Pseudoplacoid	Forked pseudoplacoid	Auricillica	Trichoidea	Basiconica	Coeloconica
R. nubila	major	major	-	present	-	present
E. semipurpurella	-	-	major	present	-	present
L. capitella	-	-	-	major	present	present
B. anynana	-	-	-	major	present	present
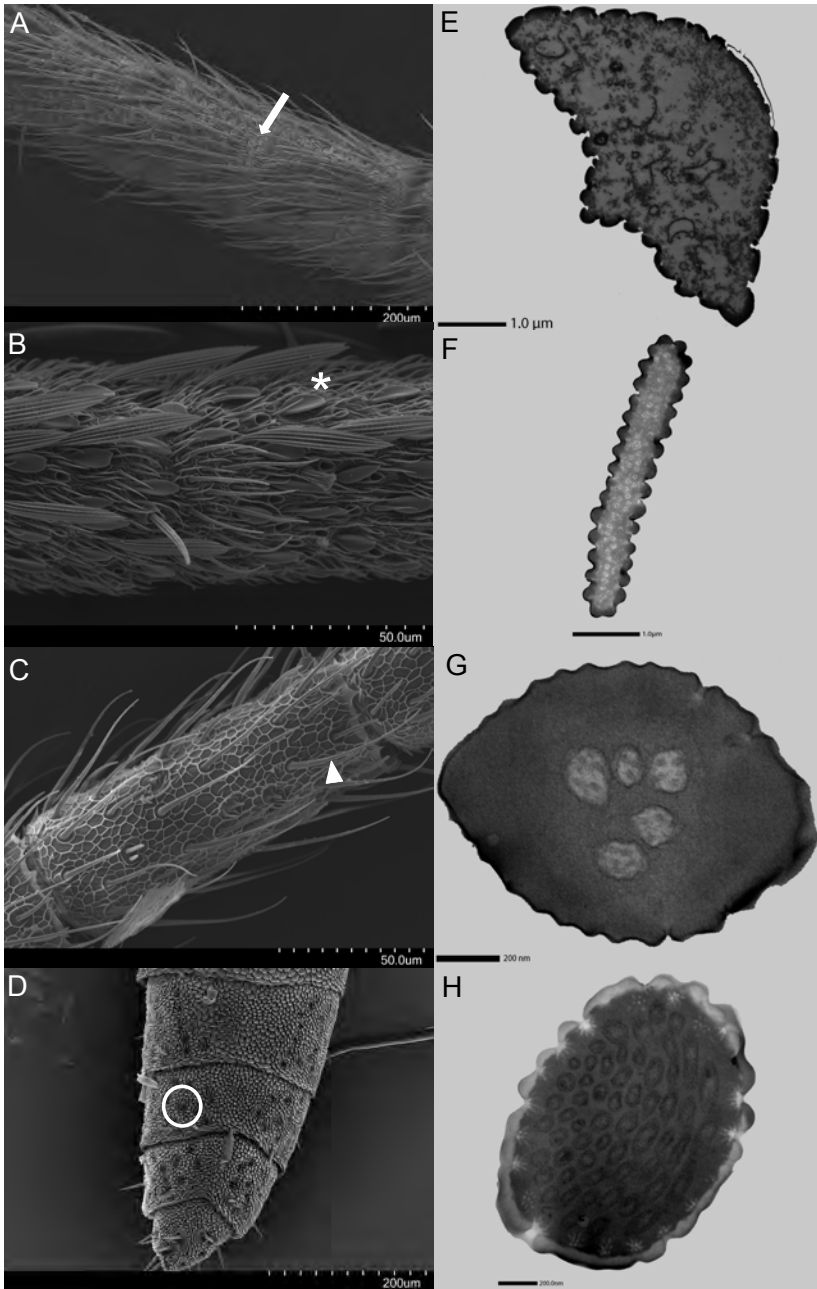
Figure 9: Scanning and transmission electron microscopy images from *Rhyacophila nubila, Eriocrania semipurpurella, Lampronia capitella* and *Bicyclus anynana*. Overview and major sensilla type of *R. nubila, E. semipurpurella, L. capitella* and *B. anynana* antennae, A) arrow- mushroom-like pseudoplacoid, B) *-sensilla auricillica, C) arrowhead- sensilla trichoidea, D) circle-sensilla basiconica (not the major sensilla type). Cross-section of E) mushroom-like pseudoplacoid, F) sensilla auricillica, G) sensilla trichoidea and H) sensilla basiconica, respectively.

Antennal lobe organisation

The approximate numbers of glomeruli in the antennal lobes were counted from the 3D voltex images and orthoslice (three orthogonal slices through X, Y, or Z axis of the volume). Number of replicates include both antennal lobes (AL) from a single or multiple individuals. Male and female *R. nubila* AL were found to have a total of ~29±1 glomeruli (n=2) and ~30±1 glomeruli (n=2), respectively (Fig. 10A and B). *E. semipurpurella* male AL contained a total of ~34±2 glomeruli (n=2) (Fig. 10C). Both *R. nubila* and *E. semipurpurella* ALs contain a lower number of glomeruli compared to *L. capitella* and derived moths, which have few enlarged glomeruli (MGC) and many small glomeruli (Hansson et al. 1991; Nakimi et al. 2014; Montgomery and Otto 2015) (Fig. 10A, B and C). Interestingly, large glomeruli similar to those forming MGC were found in both males and females (not clear) of *R. nubila* and males of *E. semipurpurella* (female samples could not be obtained) (Fig. 10A, B and C). However, at this point it is hard to compare the male and female AL in *R. nubila* with our current volume rendering images. Hence, more replicates and detailed AL reconstruction will be necessary to interpret these data. In *E. semipurpurella,* it has been shown that its PRs likely have evolved from plant volatile detecting ORs (Yuvaraj et al. 2017). Hence, it is also possible that, to adjust the shift from plant volatile detecting ORs to pheromone detecting PRs, the existing large glomeruli dedicated to plant volatiles may have been recruited for processing pheromone information. It is also an advantage to avoid rewiring the connection between the pheromonal glomeruli and the PR-expressing OSNs.

Male and female of *L. capitella* had a total of ~48±2 glomeruli (n=2) and ~50±2 glomeruli (n=2), respectively. The male MGC of *L. capitella* appears as cluster of three large glomeruli (Fig. 10D), the first evidence for the presence of well-defined MGC in a non-ditrysian moth (Fig. 10D). The number of glomeruli in the MGC correlates with the components of the pheromone blend (Hansson et al. 1991; Nakimi et al. 2014). The morphological changes in the MGC are likely correlated with the number of pheromone components and their behavioral importance (Hansson et al. 1991; Namiki et al. 2014). In Bombycidae moths, it has been shown that changes in pheromone components from two components to a single component may alter the volume of the MGC (Hansson et al. 1991; Nakimi et al. 2014). The pheromone blend of *L. capitella* female consist of three components and all three components showed antennal response in males (Löfstedt et al. 2004).
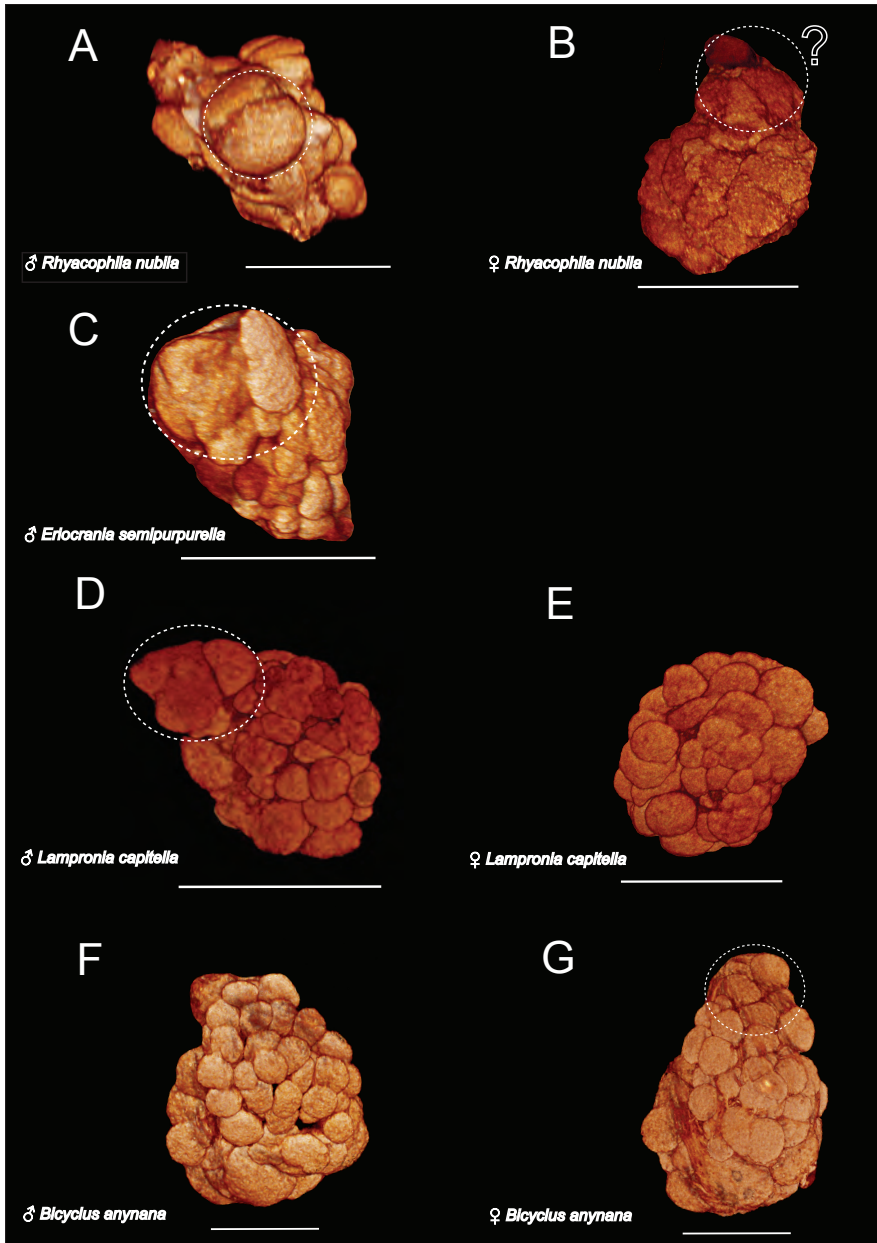
Figure 10. Anatomical organization of the antennal lobes (ALs) obtained from whole mount preparations. The antennal lobe was volume rendered and the surrounding parts were removed from the image stack. Anterior view of the AL, left panel (left AL- male) and right panel (right AL- female), Scale bars= 100 μm. A-B) male and female AL of *Rhyacophila nubila*, C) Male AL of *Eriocrania semipurpurella*, note: no female AL obtained. D-E) male and female AL of *Lampronia capitella*, F-G) male and female AL of *Bicyclus anynana.* Presence of proposed Macro glomerular complex (MGC) indicated with dotted circles.

MGC was found only in the males of *L. capitella* but not in the female AL (Fig. 10D and E). In the antennal transcriptome studies of *L. capitella,* I found two ORs (OR6 and 8) expressed higher in males than females and both of them responded to the pheromone compounds in the functional assay (Chapter II). Hence, it could be possible that the large number of OSN expressing these highly expressed OR converge to form the large glomeruli to process the pheromone related information. In many moths, it has been shown that that pheromone detection is restricted to the MGC in the antennal lobes of male moths (Hansson et al. 1991; Berg et al. 1998; Nakimi et al. 2014; el Jundi et al. 2009).

Male and female *B. anynana* ALs consist of ~62±2 glomeruli (n=4), and ~60±2 glomeruli (n=4), respectively (Fig. 10F and G). With the current volume rendering images MGC-like large glomeruli found only in female AL of *B. anynana*, which lie in a similar position as the moth MGC (i.e. more proximally to the antennal nerve input) that has shown to be involved in pheromone detection (Fig. 10F and G). However, no sexually dimorphic MGC has been described in the many previous studies on other Nymphalidae (Heinze and Reppert 2012; Carlsson et al. 2013; Montgomery and Otto 2015; Montgomery et al. 2016), but there was sexual dimorphism in the volume of MGC in *Godyris zavaleta* (Montgomery and Otto 2015). Butterflies are supposed to rely more heavily on visual cues due to their diurnal lifestyle. Also, butterflies apparently lost the long range chemical communication using sex pheromone compounds and rather use smaller spatial range communication with male produced pheromones (Andersson et al. 2007; Nieberding et al. 2008; Carlsson et al. 2013). However, previous laboratory behavioral assays on *B. anynana* demonstrated that both chemical and visual cues play an important role in female choice (Costanzo and Monteiro 2007). Electrophysiological studies showed that male and female antennae of *Pieris napi*, and female antennae of *B. anynana* respond to the male produced pheromone compounds (Anderson et al. 2007; Nieberding et al. 2008). However, the preliminary data set on *B. anynana* ALs did not provide any clear conclusion about the presence of dimorphic MGC or their potential role in pheromone detection.

Here only preliminary results from the immunocytochemistry studies of the AL were presented and discussed. However, more robust analysis of the current data and future studies on closely related moth species may add knowledge on the glomerular organization in the MGC and their relationship with different pheromone components used. To understand the association between sex pheromone detecting sensory neurons and the MGC more neuron tracking studies of pheromone sensing neurons are necessary.

Conclusions and future perspectives

Antennal transcriptome study yielded the first set of chemosensory genes identified from a trichopteran species and two basal lepidopteran moths. The phylogenetic analysis shows that, PRs and PBPs in Lepidoptera evolved in relation to the transition to Type I pheromones. However, we have only analyzed antennally expressed chemosensory genes and there might be more genes found in the genomes. The amino acid sequences of SNMP1 orthologous of more derived moths were more similar to each other than to SNMP1 orthologous of basal Lepidoptera and Trichoptera suggesting their importance in moth pheromone detection. However, the suggested role of PBPs and SNMPs need to be validated by functional studies of these chemosensory proteins and additional transcriptome analysis of closely related species.

The phylogenetic and functional characterization studies on ORs from *E. semipurpurella* shows that: 1) The PRs that responded to Type 0 pheromone compounds are also weakly responding to structurally similar plant volatile compounds, indicating that the PRs for Type 0 pheromones have evolved from ORs that involved detecting plant volatiles. 2) EsemOR1 that falls at the base of the PR clade and responds specifically to the plant volatile compound β-caryophyllene suggests a hypothesis that PRs for Type I pheromones may also have evolved their function from plant volatile detecting ORs. None of the ORs from *R. nubila* and *E. semipurpurella* group within the classical PR clade that consist of conserved receptors and some functionally characterized PRs for Type I and II pheromone compounds. However, three functionally active PRs were found from *L. captella* that group next to the previously defined PR clade which allows us to extend the PR clade, from a functional perspective, by including these receptors. On the other hand, the clade that contains sex-biased ORs from other moth species, group in between the two *L. capitella*-specific OR clade that contain functionally characterized receptor for Type I pheromone compounds suggesting that the receptors in the sex-biased receptor clade might very well respond to Type I sex pheromone compounds.

The antennal morphological data suggest that there was a major shift in sensillum types during the divergence of Lepidoptera from their sister order Trichoptera. *R. nubila* and *E. semipurpurella* belong to Trichoptera and basal Lepidoptera, respectively, and both contain similar sensillum types. But, the non-dirtysian moth (still basal) *L. capitella* has sensilla types that resemble more those of derived moths than of the basal moths, which may be an adaptation to detect certain

ecologically important volatile cues. We do not yet know the function of different types of sensilla found in Trichoptera and basal Lepidoptera, except, that sensilla auricillica in *E. semipurpurella* are tuned to Type 0 pheromone compounds and sensilla trichoidea are tuned to Type I pheromone compounds in many derived moths. Future electrophysiological studies on trichopteran sensilla types will reveal their function in olfaction.

It is important to keep in mind that the current study only identified genes from antennal transcriptome, hence it is also possible that the chemosensory genes that are expressed in other tissues or life stages may possibly be missing in the antennal transcriptome sequencing, e.g. OR that may be part of the PR clade but not found in the transcriptomic data. Thus, future genome sequencing studies are needed to better understand the evolution of the chemosensory gene families.

The immunocytochemistry studies revealed that the number of ORs found in the antennal transcriptome studies roughly correlate with the number of glomeruli present in the AL. Interestingly, large glomeruli similar to the macro glomerular complex (MGC), that process sex pheromone information in males of derived moths, were present in males of *E. semipupurella* and both sexes of *R. nubila.* These MGC-like glomeruli may be involved in processing behaviorally important volatiles such as host cues or pheromone compounds. However, MGC was only present in the male AL of *L. capitella*, which could be because sex pheromone communication involves female produced pheromone compounds detected by males, which is common across ditrysian moth species. Whereas, in *B. anynana* males produce sex pheromone compounds that are used for short-range courtship behaviour. Hence, the presence of MGC-like glomeruli found in the females of *B. anynana* may have dedicated to detect the male-produced pheromone compounds. Future studies on the neuronal pathway for sex pheromone processing in basal moths and butterflies should provide insights into the function and evolution of these structures.

The functional studies suggest that PRs for Type 0 and I pheromone compounds may have evolved from plant volatile detecting ORs. However, additional studies on ORs from basal lepidopteran lineages are necessary to test this hypothesis. On the other hand, the motifs in these basal moth species are not so conserved. Hence, to understand the role of the PR motifs in pheromone detection or receptor specificity, site-directed mutagenesis studies on the PR motifs of the ORs from the basal Lepidoptera are necessary. The mutagenesis studies will also help to identify what alterations occurred in the OR sequences during the recruitment of plant volatile detecting ORs to detect the sex pheromone compounds.

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Popular science summary- English

The sense of smell is important for animals including humans, dogs, insects and many other animals. Particularly, in insects such as moths and flies, the sense of smell is predominantly used to find mating partners, food, a place to lay eggs, and also to avoid enemies. Pheromones are chemicals produced and released by an organism that change the behavior or physiology of another individual of the same species. In moths, typically females produce pheromone compounds to attract males over a long distance. Insects have antennae that have a similar role as the human nose, which is to sense the odor molecules present in the environment.

The surface of the antennae houses morphologically differentiated structures, often hair-like, called sensilla. The sensilla contain odorant receptors (ORs), i.e. proteins specifically tuned to detect certain odor molecules. There are additional players involved in the odor detection process also located within the sensilla, such as the odorant receptor co-receptor (Orco). The combination of OR and Orco, located in the dendrites (short, branched extensions of a nerve cell) of the olfactory sensory neurons (OSNs), detects and translates the chemical information into neuronal signals. The nerve of the OSNs is connected to the antennal lobe (AL). In the AL incoming nerves are organized in glomeruli, where the first processing of the odor information takes place. The processed information in the AL is afterwards sent to higher processing centers and this might lead to behavioral output. In derived moths (relatively recently evolved), the so called macro glomerular complex (MGC) in the AL is exclusively dedicated to process pheromone-related information.

A specific set of receptors are used to detect different compounds, for example pheromone receptors (PRs) are used to detect sex pheromone compounds and other ORs detect other ecologically important volatile compounds. When a new pheromone signal evolves, a matching evolution of the receptors in the responder is required to maintain mutual communication between the signal-sender (female) and signal-responder (male). Most research on pheromone production and perception in terms of behavior and physiology have focused on derived Lepidoptera (moths and butterflies), but very little is known about evolution of pheromone receptors in basal moths (more ancestral in evolutionary sense). In order to understand the evolution of pheromone receptors in moths it is important to study the basal moths.

In this thesis, I analyse basal insect species using two different pheromone types and located in interesting positions in the phylogenetic tree of insects. Caddisflies (Trichoptera) is the sister group of Lepidoptera (moths and butterflies). The caddisfly (*Rhyacophila nubila*) and the basal leaf miner moth (*Eriocrania semipurpurella*) both use pheromone compounds classified as Type 0, a type which chemically resembles many odorants of plants. The currant shoot borer moth (*Lampronia capitella*) and a butterfly, the squinting bush brown (*Bicyclus anynana*) both uses another type of pheromone compounds called Type I, long-chain compounds with acetates, alcohols or aldehydes at one end. To obtain a better picture of evolution of chemoreception in Lepidoptera and Trichoptera, I compare different chemosensory components of these four species. But, for the butterfly, the squinting bush brown, only the antennal morphology and AL architecture are studied.

I use antennal tissue to identify the chemosensory genes that are involved in chemosensation. I use human embryonic kidney cells to express the ORs and functionally test their responses to pheromone and plant compounds. With the help of scanning and electron microscopy techniques I document the morphology and ultrastructure characteristics of different types of sensilla in the four species. Immunocytochemistry studies (staining technique used to visualize specific parts of the tissue) are used to study the glomerular architecture of the antennal lobe.

The functional characterization studies of ORs from *E. semipurpurella* identified the first receptors for Type 0 pheromone compounds. The Type 0 pheromone receptors also responds weakly to structurally similar plant volatiles. This finding suggests that, the receptors for pheromone detection in *E. semipurpurella* have evolved by modifying the plant odorant receptors which possibly were used to find the host plants. Functional studies on one of the most basal group of moths, *L. capitella* receptors lead to the first characterization of pheromone receptor that responds to Type I pheromone compounds.

The morphological study of sensillum types found in Trichoptera and Lepidoptera reveals that there has been an evolutionary shift in major sensillum types. For the first time, I report MGC-like glomeruli of an insect using a Type 0 pheromone. Also, in *L. capitella* and *B. anynana* MGC is present only in the sex for which the sex pheromone detection is important.

In this thesis, the first pheromone receptors are characterized from basal moths. The results suggest that pheromone detecting receptors in basal moths have evolved from plant odour detecting receptors. The current results increase our knowledge on the evolution of sex pheromone reception in moths. Further work on the chemosensory gene families from more basal moths should provide deeper insight into the evolution of chemoreception in moths.

Populär sammanfattning på svenska

Resultaten i denna avhandling ökar vår kunskap om hur evolutionen av feromondetektering hos fjärilar gått till.

Luktsinnet är viktigt för de flesta djur, inklusive människor, hundar, insekter och många andra. Hos insekter, särskilt nattfjärilar och flugor, används luktsinnet för att hitta parningspartners, föda, äggläggningsställen och att undkomma fiender.

Feromoner är kemikalier som produceras och avges av en organism och som ändrar beteende eller fysiologi hos en annan individ inom samma art. Hos nattfjärilar är det för det mesta honan som avger feromoner, för att locka till sig hannar, ofta från långt håll. Hos insekterna är det antennen som har den funktion som vår näsa har, nämligen att känna dofter.

På antennens yta ryms en mängd hårliknande sensiller. Dessa sensiller rymmer doftreceptorer, som är proteiner inställda på att detektera specifika molekyler. Det finns ytterligare aktörer som är involverade i doftigenkänningsprocessen, och som är lokaliserade i sensillerna, och det är hjälp-receptorer, dvs. medmottagare till doftreceptorerna. Båda sitter i dendriterna, som är korta förgrenade nervcellsutskott. Kombinationen av doftreceptorer och dess hjälp-receptorer översätter doftinformationen som mottages och skickar in den i nervcellen. Nervcellen är av en speciell typ, som kallas doftigenkänningsnervcell. Den går från antennen in i en antennlob. I antennloben är de inkommande nervcellerna organiserade i något som kallas glomerulus, där den första bearbetningen av inkommande doftdata sker. Hos nattfjärilar som utvecklats relativt sent ur ett evolutionärt perspektiv finns i glomerulus särskilda ansamlingar, s. k. makroglomeruluskomplex, som enbart bearbetar feromonrelaterad information. Den bearbetade informationen i glomerulus skickas sedan vidare till högre nivåer i hjärnan, för vidare bearbetning. Detta kan slutligen leda till att utlösa ett beteende, t. ex. att börja flyga.

En särskild uppsättning av receptorer används för att känna igen olika ämnen, exempelvis används feromonreceptorer för att känna igen feromonämnen. Andra uppsättningar av receptorer används för att känna igen andra ekologiskt viktiga doftämnen, t. ex. värdväxtdofter. När en ny feromonsignal utvecklas, måste en motsvarande matchning av receptorerna hos mottagaren utvecklas, för att

upprätthålla kommunikationen mellan den avsändande honan och den mottagande hannen.

Den mesta forskningen om feromonproduktion och -perception kopplat till beteende och fysiologi har hittills mest fokuserat på nattflyn och dagfjärilar. Mycket lite är känt om evolutionen hos feromonreceptorerna hos mer "primitiva" fjärilar, dvs. mer ursprungliga ur evolutionär synvinkel. För att förstå hur feromonreceptorerna utvecklats hos fjärilarna (ordningen Lepidoptera) är det viktigt att studera även dessa "primitiva" fjärilar.

I denna doktorsavhandling analyserar jag fyra insektsarter, som använder två olika slags feromontyper och som återfinns i intressanta positioner i insekternas fylogenetiska träd. Nattsländor (ordningen Trichoptera) är systergrupp till fjärilar (ordningen Lepidoptera). Nattsländan *Rhyacophila nubila* och den "primitiva" fjärilen vårpurpurmal (*Eriocrania semipurpurella*) använder båda en feromontyp som kallas Typ 0, en typ som kemiskt påminner om många växtsubstanser. Vinbärsknoppmal (*Lampronia capitella*) och den afrikanska dagfjärilen *Bicyclus anyana* använder däremot en feromontyp som kallas Typ I, som utgörs av mer långkedjiga föreningar med acetater, alkoholer eller aldehyder i ena molekyländen. För att få en bättre förståelse av hur dessa fjärilars och nattsländors luktorgan utvecklats jämför jag olika organ hos de fyra arterna. Men när det gäller *Bicyclus anyana* har bara antennmorfologi och antennlobsstrukturen studerats.

Jag använder antennvävnad för att identifiera de kemosensoriska gener som styr kemisk sensitering. Till hjälp har jag HEK-celler. HEK är en förkortning för *Human Embryonic Kidney*, det vill säga celler från njuren hos mänskliga embryon. Med hjälp av HEK-celler kan jag uttrycka doftreceptorerna och testa deras reaktion på olika feromoner och växtdofter. Svepelektronmikroskopi och transmissionselekronmikroskopi används för att i mycket hög förstoring dokumentera morfologiska och strukturella detaljer hos sensillerna hos de fyra arterna. Immuncytokemiska metoder såsom infärgning används för att visualisera nervcellsuppbyggnaden i antennloben.

Den funktionella karakteriseringen av doftreceptorerna hos vårpurpurmalen identifierar för första gången receptorer som detekterar feromonkomponenter av Typ 0. Dessa receptorer reagerar till viss del även på strukturellt likartade växtdofter. Detta tyder på att feromonreceptorerna hos vårpurpurmalen har utvecklats genom att växtdoftreceptorer, som användes för att hitta värdväxter, modifierats till att bli feromonreceptorer. De funktionella studierna av receptorerna hos vinbärsknoppmalen visar å andra sidan för första gången feromonreceptorer hos den mest "primitiva" fjärilsgruppen som reagerar på feromoner av Typ I.

De morfologiska studierna av sensilltyperna hos nattsländan och nattfjärilar visar att det inträffat en stor evolutionär förändring, ett så kallat skifte, hos viktiga sensilltyper. För första gången visas att en insekt som använder feromon av Typ 0 har glomeruli som innehåller makroglomeruluskomplex. Jag visar också att hos vinbärsknoppmalen *L. capitella* och hos *B. anyana* förekommer makroglomerulusliknande strukturer bara hos det kön för vilket feromondetektering är viktigt, inte hos det andra könet.

Fortsatt forskning om genfamiljer som styr reaktioner på kemiska signaler hos de primitiva fjärilarna skulle ge ytterligare insikter i hur denna evolution gått till.

பிரபல தமிழ் அறிவியல் சுருக்கம்

மனிதர்கள், நாய்கள், பூச்சிகள் மற்றும் பல விலங்கினங்களை சார்ந்த மிருகங்களுக்கு வாசனை உணர்வு முக்கியமாகும். குறிப்பாக, பூச்சிவகைகளைச் சார்ந்த ஈக்கள் மற்றும் பழப்பூச்சிகளில், வாசனை உணர்வானது இனப்பெருக்க ஜோடிகளைக் கண்டறியவும், உணவைக் கண்டறியவும், முட்டை இடுவதற்கு சரியான இடத்தைக் கண்டறியவும், எதிரிகளிடமிருந்து தப்பிக்கவும் பயன்படுகிறது. பெரோமோன்கள் (இனக்கவர்ச்சி ஹார்மோன்) என்பது, ஒரு உயிரினத்தின் நடத்தை அல்லது உடலியல் ஆகியவற்றை மாற்றும் அதே வகை உயிரினத்தால் உருவாக்கப்பட்டு வெளியிடப்படும் இரசாயனங்கள் ஆகும். அந்துப்பூச்சி (moths) இனங்களில், பொதுவாக பெண்பூச்சிகள் பெரோமோன் கலவைகளை கொண்டு வெகு தொலைவில் உள்ள ஆண் பூச்சிகளைக் கவர்கிறது. பூச்சிகளானது மனிதமூக்கு போன்ற ஒத்த உணர்வு நீட்சிகளைக் கொண்டு, சுற்றுப்புறச்சூழலில் உள்ள வாசனை மூலக்கூறுகளை உணர்கிறது.

உணர்வு நீட்சிகளின் மேற்பரப்பு பெரும்பாலும் வெவ்வேறு உருவமுடைய முடி போன்ற சென்சில்லா (sensilla) எனப்படும் அமைப்பைக் கொண்டுள்ளது. குறிப்பிட்ட வாசனை மூலக்கூறுகளைக் கண்டறிய சென்சில்லாவில் (sensilla) உள்ள வாசனை ஏற்பிகளானது பயன்படுகிறது. வாசனைகள் உணர்வதில் சென்சில்லாவில் (sensilla) உள்ள வாசனை ஏற்பிகள் (ORs) மற்றும் வாசனை ஏற்பிகளின் இணை-ஏற்பிகள் (Orco) போன்ற கூடுதல் அமைப்புகள் உள்ளன. சிறு நரம்பு இழையிலுள்ள (dendrites) வாசனை ஏற்பிகள் (ORs) மற்றும் அதன் இணை ஏற்பிகளின் (Orco) சேர்க்கையானது வேதியியல் தகவல்களை நரம்பு சமிக்ஞைகளாக மாற்றுகிறது. சிறு நரம்பு இழையானது (dendrites) வாசனை உணர் நரம்புகளோடு (olfactory sensory neurons, OSNs) இணைந்துள்ளது. வாசனை உணர் நரம்பானது (OSNs) உணர்வு நீட்சி மடலொடு (antennal lobe, AL) இணைக்கப்பட்டுள்ளது. உணர்வு நீட்சி மடல்களுக்கு வரும் நரம்பானது வடி முடிச்சு (glomeruli) மூலம் ஒழுங்குபடுத்தப்பட்டு, வாசனை தகவல்களின் முதல் செயல்பாடுகள் வடி முடிச்சு மூலம் செயல்படுத்தப்படுகிறது. உணர்வு நீட்சி மடலில் செயலாக்கப்பட்ட தகவல் பின்னர் மூளைக்கு அனுப்பப்பட்டு, நடத்தை வெளியீட்டிற்கு வழிவகுக்கப்படுகிறது. பரிணாமம் அடைந்த பூச்சிகளில் உணர்வு நீட்சி மடலில் (AL) உள்ள மேக்ரோ குரோமருளர் காம்ப்ளெக்ஸ் (MGC) என்று அழைக்கப்படுவது பிரத்தியேகமாக பெரோமோன் தொடர்பான தகவலுக்காக அர்ப்பணிக்கப்பட்டதாகும்.

ஒரு குறிப்பிட்ட வகை ஏற்பிகளானது, வெவ்வேறு சேர்மங்களை கண்டுபிடிக்கப் பயன்படுகிறது. உதாரணமாக பெரோமொன் ஏற்பிகள்(PRs) பாலியல் பெரோமோன் கலவைகள் கண்டுபிடிக்கவும், மற்றும் வாசனை ஏற்பிகள் (ORs) முக்கிய சுற்றுச்சூழல் சேர்மங்களைக் கண்டறியவும்

பயன்படுகிறது. பரிணாமநடத்தை மற்றும் உடலியல் ஆகியவற்றில் பெரோமோன் உற்பத்தி மற்றும் உணர்வின் மீதான அதிகமான ஆராய்ச்சி லீபிடொப்டெரா பூச்சிவகைகள் (அந்துப்பூச்சிகள் மற்றும் பட்டாம்பூச்சிகள்) மேற்கொள்ளப்பட்டுள்ளது. ஆனால் பரிணாம வளர்ச்சியில் பின் தங்கிய பூச்சிகளில் பெரோமோன் ஏற்பிகளின் பரிணாமத்தைப் பற்றிய அறிதல் குறைவாக உள்ளது. பூச்சிகளில் பெரோமோன் ஏற்பிகளின் பரிணாமத்தை புரிந்து கொள்வதற்கு பரிமாண வளர்ச்சியில் பின் தங்கிய பூச்சிகளைப் படிப்பது முக்கியம்.

இந்த ஆய்வில், நான் இரு வேறு பெரோமொன் வகைகளை பயன்படுதும், பரிணாம-மரத்தில் சுவாரசியமான இடத்தில் உள்ள நான்கு பூச்சிவகைகளை ஆய்விற்காக பயன்படுத்தினேன். காடிஸ்ஃபிளீஸ்-பூச்சிவகை (டிரிகோப்ட்டரா) என்பது லீபிடொப்டெராவின் (அந்துப்பூச்சிகள் மற்றும் பட்டாம்பூச்சிகள்) சகோதரி குழுவாகும். காடிஸ்ஃபிளீஸ்-பூச்சிவகை (ரையாக்கோபிலா நுபிலா) மற்றும் இலை சுரங்க அந்துப்பூச்சி (எரியோகிரானியா செமிப்பர்புல்லா) இரண்டும், வகை-0 என வகைப்படுத்தப்படும் பெரோமோன் கலவைகளைப் பயன்படுத்துகின்றன. வகை-0 பெரோமோன் மூலக்கூறு என்பது வேதியியல் ரீதியாக தாவரங்களின் பலவாசனை மூலக்கூறுகளை ஒத்திருக்கிறது. திராட்சை துளைப்பான் அந்துப்பூச்சி (லம்ப்ரோனியா காபிடெல்லா) மற்றும், ஒரு பட்டாம்பூச்சி (பைசைக்கிளஸ் அனினானா) இரண்டும் வகை-I எனப்படும் பெரோமோன் கலவைகளைப் பயன்படுத்துகிறது. வகை-I பெரோமோன் மூலக்கூறு என்பது நீண்ட சங்கிலி மூலக்கூறுகளில் அசிட்டேட்ஸ், ஆல்கஹால்ஸ் அல்லது அல்டிஹைட்ஸ் குழுவைக் கொண்டிருக்கும். மேற்குறிப்பிட்ட நான்கு வகையான பூச்சிகளின் வேதியேற்றலின் (chemoreception) பரிணாம வளர்ச்சியின் முடிவினை பெற, பல்வேறு வகையான வேதிஉணர்வு (chemosensory) கூறுகள் ஒப்பிடப்பட்டன. ஆனால், பட்டாம்பூச்சியில், உணர்வு நீட்சி உருவமைப்பு மற்றும் உணர்வு நீட்சி மடல் (AL) கட்டமைப்பு மட்டுமே ஆய்வு செய்யப்பட்டது.

நான் உணர்வு நீட்சிகளின் திசுவை பயன்படுத்தி வேதிஉணர்வாற்றல் (chemosensation) தொடர்புடைய வேதிஉணர்வு மரபணுக்களை அடையாளம் கண்டறிந்தேன். நான் மனித சிறுநீரக செல்களைப் பயன்படுத்தி பெரெமோன் ஏற்பிகளின் செயல்பாட்டைக் கண்டறிந்தேன். ஸ்கேனிங் மற்றும் மின்அணு நுண்ணோக்கிகளின் உதவியுடன் நான் நான்கு வகையான பூச்சிகளின் உணர்வு நீட்சி உருவமைப்பு, தோற்ற நிலை மற்றும் உள் கட்டமைப்புப் பண்புகளை ஆவணப்படுத்தியிருக்கிறேன். உணர்வு நீட்சி மடலின் வடிமுடிச்சு கட்டமைப்புகளை ஆய்வு செய்ய, இம்யூனோசைட்டோகெமிஸ்ட்ரி ஆய்வுகள் (திசுவின் குறிப்பிட்ட பாகங்களைக் நிறமிடுவதற்கு பயன்படுத்தப்படும் நுட்பம்) பயன்படுத்தப்பட்டன.

எ. செமிபர்புரல்லாவின் வாசனை ஏற்பியின் செயல்பாட்டு பண்பியல் ஆய்வுகள், வகை-0 பெரோமோன் சேர்மங்களுக்கான முதல் ஏற்பியை அடையாளம் காட்டின. வகை-0 பெரோமோன் ஏற்பிகள் வேதியியல் ரீதியாக பெரோமோன் போன்ற கட்டமைப்புக் கொண்ட பல தாவரங்களின் வாசனை மூலக்கூறுகளுக்கு குறைவான உணர்வை வெளிப்படுத்தின. இந்த கண்டுபிடிப்பு, எ. செமிபர்புரல்லவில் உள்ள பெரோமோன் ஏற்பிகள் தாவரங்களின் வாசனையை உரர பயன்படுத்தப்படும் ஏற்பிகளில் இருந்து பரிணாம வளர்ச்சி மூலம் உருவாகியிருக்கின்றன என்பதை காட்டுகிறது. எல்கேபிடெல்லா ஏற்பிகளின் செயல்பாட்டு ஆய்வுகள், பெரும்பாலான பரிணாம வளர்ச்சியில் பின்தங்கிய அந்துப்பூச்சிகளின் முதல் வகைப்படுத்தப்பட்ட வகை-I பெரோமொன் ஏற்பியை கண்டறிய வழிவகுத்தது.

ட்ரிக்கோபிடீர மற்றும் அந்துப்பூச்சிகளின் உருவ ஆய்வு, சென்சில்லா வகைகளில் ஒரு மிகப்பெரிய பரிணாம மாற்றம் ஏற்பட்டுள்ளது என்பதை தெளிவுபடுத்துகிறது. முதல் முறையாக, 0-வகை பெரோமோனைப் பயன்படுத்தும் பூச்சியில் மேக்ரோக்ரோமருளர் காம்ப்ளைஸ போன்ற அமைப்பு கண்டுபிடிக்கப்பட்டுள்ளது. மேலும், எல். கேபீடல்லா மற்றும் பை. அநினானாவில் மேக்ரோக்ரோமருளர் காம்ப்ளக்ஸ் (MGC), பெரோமோன்களைக் கண்டறியும் பாலினத்தில் (ஆண்- எல். கேபீடல்லா மற்றும் பெண்- பை. அநினானா) மட்டுமே உள்ளது.

இந்த ஆய்வில், முதல் பெரோமோன் ஏற்பிகள் பரிணாம வளர்ச்சியில் பின் தங்கிய அந்துப்பூச்சிகளிலிருந்து வகைப்படுத்தப்பட்டன. அந்துப்பூச்சிகளில் பெரோமோன்களைக் கண்டறியும் ஏற்பிகள், அதன் தாவர வாசனை உணரும் ஏற்பிகளில் இருந்து பரிணாம வளர்ச்சியடைந்துள்ளது என்பது கண்டறியப்பட்டது. முதல் முறையாக பரிணாம வளர்ச்சியில் பின்தங்கிய பூச்சிகளில் இருந்து புதிய வாசனை உணர்வு சம்பந்தப்பட்ட ஜீன்களையும், அதன் நுகரும் சென்சில்லாவில் ஏற்பட்டுள்ள பெறும்பான்மையன மாற்றத்தையும் கண்டறிந்துள்ளேன். தற்போதைய முடிவுகள் அந்துப்பூச்சிகளில் பாலியல் பெரோமோன் உணர்வின் பரிணாமம் பற்றிய நமது அறிவை அதிகரிக்கின்றன. மேலும் பரிணாம வளர்ச்சியில் பின்தங்கிய அத்துப்பூச்சிகளின் வாசனை உணர்வு சம்பந்தப்பட்ட ஜீன் குடும்பங்களின் எதிர்கால ஆய்வு, அந்துப்பூச்சிகளில் வாசனை உணர்வு பரிணாம வளர்ச்சியின் ஆழமான அறிவை வழங்கும் என்பதில் ஐயமில்லை.

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தாமின் புறுவது உலகின் புறக்கண்டு
காமுறுவர் கற்றறிந் தார்.

- திருக்குறள் 399

When the learned see that their learning contributes to make all the world happy, They are pleased and pursue their learning more.

- Thirukkural no. 399

Evolution of olfaction in Lepidoptera and Trichoptera

I.   **Yuvaraj JK,** Corcoran JA, Andersson MN, Newcomb RD, Anderbrant O, Löfstedt C. 2017. Characterization of odorant receptors from a non-ditrysian moth, *Eriocrania semipurpurella* sheds light on the origin of sex pheromone receptors in Lepidoptera. *Molecular Biology and Evolution,* msx215, https://doi.org/10.1093/molbev/msx215.

II. **Yuvaraj JK,** Andersson MN, Corcoran JA, Anderbrant O, Löfstedt C. 2017. Functional characterisation of odorant receptors from *Lampronia capitella* adds functional support for the pheromone receptor clade in Lepidoptera. *Manuscript.*

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