Smells of Sociality

Insect Chemoreceptors, Sex and Habitat Cues

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Abstract

The last two decades have seen considerable research effort dedicated to understanding the molecular basis of insect olfaction. There are, however, many knowledge gaps, especially when it comes to how insects detect different olfactory stimuli from the environment. In this thesis I aim to deepen our understanding of the detection of social and environmental cues in two insect species.

Codling moth, *Cydia pomonella*, is the foremost pest of apple. Following an initial identification of the chemoreceptors in male and female antennae, we provided an extensive annotation, transcript abundance quantification and repertoire completion of each of the three main chemoreceptor gene families. These results evidenced the importance of the candidate pheromone receptors (PRs), OR1, OR6 and OR3. Then, by heterologous expression in *Drosophila melanogaster* flies we functionally characterized some of these receptors. These experiments demonstrated that CpomOR3 is highly specific and sensitive toward pear ester, a strong kairomone and pheromone synergist of codling moth, which had not previously been found in apples. These results inspired a refined analysis of apple headspace which demonstrated the presence of this kairomone in apples. Furthermore, we show a putative microbial origin of this compound. We also characterized CpomOR6 as the receptor of the pheromone antagonist codlemone acetate, and CpomOR19 as a receptor tuned to indanones. Additionally, we provide a step-by-step description of the protocol to produce and characterize insect ORs and PRs through heterologous expression in *D. melanogaster*. Finally, we identified chemoreceptors from three tortricids and predict their function based on our results from codling moth. In addition, we investigated the common fruit fly *D. melanogaster*. In this species, an olfactory gene, OR69a, is expressed as two alternatively spliced variants: OR69aA and OR69aB. Through means of single sensillum recordings (SSRs) we characterized the non-overlapping response of both variants, showing that OR69aA responds to several plant compounds, while OR69aB detects a novel, long-range female-produced pheromone.

Together our results show that insects possess elegant ways to detect species-specific signals and the habitat cues that interact with those signals.

Keywords: Cydia pomonella, *Drosophila melanogaster*, insect odourant receptors, SSRs, *Hedya nubiferana*, *Cydia fagiglandana*, *Cydia nigricana*.

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Dedication

To my family: full blood, half-blood and non-blood related, especially to the beloved memory of María González.

Intelligence is based on how efficient a species became at doing the things they need to survive.

Charles Darwin

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List of Publications

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

I William B. Walker, **Francisco Gonzalez**, Stephen F. Garczynski, and Peter Witzgall. (2016). "The chemosensory receptors of codling moth *Cydia pomonella*–expression in larvae and adults." *Scientific Reports* 6:23518.

II Jonas M. Bengtsson, **Francisco Gonzalez,** Alberto M. Cattaneo, Nicolas Montagné, William B. Walker, Marie Bengtsson, Gianfranco Anfora, Rickard Ignell, Emmanuelle Jacquin-Joly, and Peter Witzgall. (2014). "A predicted sex pheromone receptor of codling moth *Cydia pomonella* detects the plant volatile pear ester." *Frontiers in Ecology and Evolution* 2 (33).

III Alberto Maria Cattaneo, **Francisco Gonzalez**, Jonas M. Bengtsson, Elizabeth A. Corey, Emmanuelle Jacquin-Joly, Nicolas Montagné, Umberto Salvagnin, William B. Walker, Gianfranco Anfora, Peter Witzgall and Yuriy V. Bobkov. (2017). “Candidate pheromone receptors from the insect pest *Cydia pomonella* respond to pheromone and kairomone components”. *Scientific Reports* 7:41105.

IV **Francisco Gonzalez**, Jonas M. Bengtsson, William B. Walker, Maria FR Sousa, Alberto M. Cattaneo, Nicolas Montagné, Arthur de Fouchier, Gianfranco Anfora, Emmanuelle Jacquin-Joly, Peter Witzgall and Rickard Ignell. (2015). "A Conserved Odourant Receptor Detects the Same 1-Indanone Analogs in a Tortricid and a Noctuid Moth." *Frontiers in Ecology and Evolution* 3 (131).

V **Francisco Gonzalez**, Peter Witzgall, and William B. Walker. (2016). "Protocol for heterologous expression of insect odourant receptors in Drosophila." *Frontiers in Ecology and Evolution* 4 (24).

VI **Francisco Gonzalez**, Maria Sousa, Lucie Conchou, William B. Walker, Amrita Chakraborty, Maria Karlsson, Göran Birgersson, Beatrix Alsanius, Alan Knight and Peter Witzgall. "Pear ester: the host cue that was not there". Manuscript.

VII Sebastien Lebreton, Felipe Borrero-Echeverry, **Francisco Gonzalez,** Marit Solum, Erika Wallin, Federica Trona, Amelie Baschwitz, Veit Grabe, Volker Jörger, Silke Sachse, Erik Hedenström, Bill S. Hansson, Anna-Lena Gustavsson, Marie Bengtsson, Göran Birgersson, William B. Walker, Hany Dweck, Paul Becher, Peter Witzgall. (2017). "The Drosophila pheromone Z4-11Al is encoded together with olfactory cues and mediates species-specific communication. *bioRxiv* 083071

VIII **Francisco Gonzalez**, Peter Witzgall and William B. Walker. (2017). **"**Antennal transcriptomes of three tortricid moths reveal conserved chemosensory receptors for social and habitat olfactory cues". *Scientific Reports* 7:41829.

The contribution of Francisco Gonzalez to the papers included in this thesis was as follows:

I Carried out bioinformatics pipelines, gene annotation and wrote the manuscript with co-authors.

II Carried out all electrophysiological recordings, analysed the data and wrote the manuscript with co-authors.

III Planned and performed all electrophysiological recordings and assisted in data analysis.

IV Planned and carried out all experiments, data analysis and wrote the manuscript with co-authors.

V Wrote the manuscript with co-authors.

VI Planned and assisted in volatile collections, chemical analyses and performed all the electrophysiological recordings, and wrote the manuscript with co-authors.

VII Planned and performed part of the electrophysiological recordings and assisted in writing of the manuscript.

VIII Carried out all bioinformatics pipelines, gene annotation and wrote the manuscript with co-authors.

Abbreviations

AL	Antennal Lobe
cVA	11-cis Vaccenyl Acetate
GC-SSRs	Gas Chromatography Coupled to Single Sensillum Recordings
GRs	Gustatory Receptor Proteins
IRs	Ionotropic Receptor Proteins
LH	Lateral Horn
LN	Local Interneurons
MB	Mushroom Bodies
OBPs	Odourant Binding Proteins
ODEs	Odourant Degrading Enzymes
Orco	Insect Odourant Co-Receptor
ORs	Odourant Receptor Proteins
OSN	Odourant Sensory Neurons
PN	Projection Neurons
PRs	Pheromone Receptor Proteins
SNMPs	Sensory Neuron Membrane Proteins
SSRs	Single Sensillum Recordings
VOCs	Volatile Organic Compounds

1 Introduction

Insects are the most diverse group of multicellular organisms on Earth. Considering biomass and interactions with other organisms, they are by far the most successful animals in earth's history. Their success is a consequence of their ability to adapt, evident in the explosion in diversity observed since their appearance around 400 million years ago (Engel & Grimaldi, 2004).

Insects have developed sophisticated means to detect and interpret signals from biotic and abiotic components of the ecosystems which they inhabit (Bruce & Pickett, 2011; Zheng & Dicke, 2008; Bruce *et al.*, 2005; Baldwin *et al.*, 2001; Agrawal, 2000). Although senses such as sight, hearing and touch, each play an important part in the life cycles of insects, these organisms live in a "chemical world" (Schoonhoven, 1990), making smell and taste two of the most influential senses in insect evolution.

Detection and interpretation of ecologically relevant chemicals, also known as semiochemicals, has played a fundamental role in the ecological diversification of insects (Rundle & Nosil, 2005). Chemosensation mediates sexual isolation based on mate recognition, i.e. speciation based on release and detection of species-specific compounds known as pheromones (Kohl *et al.*, 2015; Greenfield, 2002; Linn & Roelofs, 1995). In addition, habitat chemical cues (kairomones) mark the right hosts, and because insects often associate their hosts with mating opportunities, host cues can also lead to speciation (Berlocher & Feder, 2002; Drès & Mallet, 2002; Filchak *et al.*, 2000). Together, pheromones and kairomones constitute the smells of sociality (Laturney & Billeter, 2013).

Chemical Ecology aims at understanding chemical-mediated interactions between insects and their habitats. Furthermore, an ultimate objetive is to use this knowledge for control of species that threaten agriculture and human health (Raguso *et al.*, 2015). Fortunately, the advent of ever more powerful

molecular techniques and computational tools has fostered the combination of chemical ecology with neuroethology allowing us to correlate the chemical-guided behaviour of insects with the neural and molecular basis of chemosensation (Ho & Riffell, 2016; Reisenman & Riffell, 2015; Purves & Lichtman, 1985). This multidiscplinary approach has allowed us to further elucidate the evolution and use of chemical signals by insects.

This thesis combines behavioural, physiological, molecular and bioinformatic techniques and studies to understand the interplay between two types of semiochemicals, sexual signals and habitat cues.

2 Background

2.1 Insect Chemosensation

2.1.1 Overview

Chemical senses are present in all organisms, from bacteria and protozoa to plants and animals. The discovery of the general principle of OR function by Linda Buck and Richard Axel was a major step forward in our understanding of how dedicated olfactory receptor proteins detect environmental chemical substances in vertebrates (Buck & Axel, 1991). Their work was awarded a Nobel Prize in Medicine and Physiology, and has enabled research on the molecular basis of olfaction. In the field of insect chemosensation, a landmark contribution came simultaneously from three independent laboratories that identified candidate olfactory receptor proteins of the fruit fly *Drosophila melanogaster* (Clyne et al., 1999; Gao & Chess, 1999; Vosshall et al., 1999). Since then, an enormous amount of work has demonstrated the importance of multiple families of proteins underlying insect chemosensation.

2.1.2 Chemosensory Gene Families

Gustatory (GRs) and odourant receptors (ORs), are proteins expressed in the membranes of chemosensory neurons housed in cuticular hair-like structures called sensilla (Gadenne *et al.*, 2016; Benton, 2015; Depetris-Chauvin *et al.*, 2015; Ray, 2015; Missbach *et al.*, 2014; Suh *et al.*, 2014). A further analysis of chemosensory genes has evidenced the existence of an extra family of receptors phylogenetically related to ionotropic glutamate receptors, called ionotropic receptors (IRs) (Hussain *et al.*, 2016; Benton, 2015; Rytz *et al.*, 2013).

Apart from ORs and IRs, three additional types of olfactory proteins have been found: sensory neuron membrane proteins (SNMPs), odourant binding proteins (OBPs) and odourant degrading enzymes (ODEs) (Figure 1).

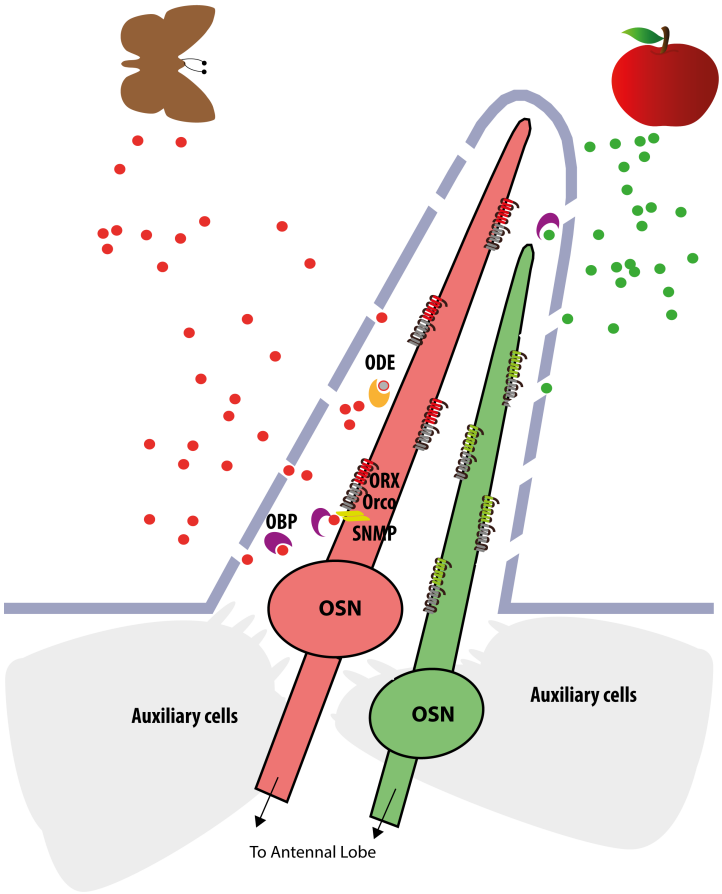

Figure 1. Main proteins in insect antennae involved in olfaction: odourant binding proteins (OBPs) attach to hydrophobic odourants and facilitate their transport to odourant sensory neuron (OSN) membranes. Sensory neuron membrane proteins (SNMPs) facilitate the movement of odourant molecules towards the chemoreceptors. Odourant receptor complex (ORX subunit + Orco) activate and amplify the response to the correspondent odourant. Odourant degrading enzymes (ODEs) deactivate the odourants once the signal is conveyed. Auxiliary cells produce OBPs, lymph and surround the OSNs.

Odourant receptors (ORs)

ORs represent the main olfactory group of proteins dedicated to the detection of volatile organic compounds (VOCs). ORs are expressed in OSNs housed inside of multiporous sensilla located in peripheral organs (antennae and palpi) (Depetris-Chauvin *et al.*, 2015; Suh *et al.*, 2014; Leal, 2013; van Loon *et al.*, 2005).

Insect ORs share several similarities with vertebrate ORs. For instance, individual OSNs generally express only one type of receptor and all OSNs expressing the same receptor converge onto the same glomerulus in the primary olfactory centre (Ache & Young, 2005; Christensen & Hildebrand, 2002). Furthermore, insects ORs also contain seven transmembrane domains. However, their topology is inverted in comparison to vertebrate ORs: the N-terminus is intracellular and the C-terminus is extracellular (Benton, 2009; Lundin *et al.*, 2007). ORs and vertebrate G-coupled proteins are not homologous, they form their own convergent gene families with distinct types of ligand-gated ion channels (Sato *et al.*, 2008; Benton *et al.*, 2006).

Another characteristic feature of insect ORs is that they depend on the interaction with a co-receptor (Orco) to function. This chaperone protein is highly conserved among all the described insect odourant receptor repertoires (Suh *et al.*, 2014; Larsson *et al.*, 2004). Although Orco does not seem to respond to a particular natural odourant, it forms heteromeric complexes with paired odour-specific ORs (ORX subunits) for odour signal transduction, in addition to participating in proper localization and maintenance of the ORX subunits in the membranes of OSNs dendrites (Nolte *et al.*, 2016; Mukunda *et al.*, 2014; Wicher *et al.*, 2008; Benton *et al.*, 2006; Larsson *et al.*, 2004).

Apart from the Orco subunit, insect ORs are highly divergent in terms of sequence similarity and number per species (Suh *et al.*, 2014). OR repertoires reflect the chemical space of the ecosystems which insects inhabit (Bohbot & Pitts, 2015). Therefore, each insect olfactory repertoire contains receptors that are extremely efficient in discriminating the specific chemical cues that convey information about food sources, oviposition sites, toxic compounds and other individuals of the same species (Depetris-Chauvin *et al.*, 2015). For this reason, the number of ORs varies among insects. Social insects such as the honeybee (*Apis mellifera*) and eusocial ants have 170 and 400 ORs, respectively, whereas mosquitoes, fruit flies and moths typically contain fewer than 100 ORs (Bohbot & Pitts, 2015; Depetris-Chauvin *et al.*, 2015).

Specificity and sensitivity of insect ORs vary among receptors. Pioneering studies on receptor tuning indicated that the fruit fly ORs were broadly tuned to multiple ligands (Hallem & Carlson, 2006; Kreher *et al.*, 2005; Hallem *et al.*, 2004), but recent evidence shows the existence of several ORs that are

narrowly tuned to highly relevant semiochemicals (Liu *et al.*, 2014; Ronderos *et al.*, 2014; Mathew *et al.*, 2013; Zhang *et al.*, 2013; Stensmyr *et al.*, 2012).

Receptors dedicated to pheromones (PRs) have received particular attention, especially in moths (Liu *et al.*, 2014; Montagné *et al.*, 2012; Wanner *et al.*, 2010b; Party *et al.*, 2009; Nakagawa *et al.*, 2005). Functional characterization, or deorphanization, of PRs holds promise for the development of strategies that might inhibit insects from smelling their conspecifics (Andersson & Newcomb, 2017).

Deorphanization of insect ORs has been facilitated by the development of *in vitro* and *in vivo* platforms that allow testing the ligand affinity of individual receptors (Wang *et al.*, 2016). *In vitro* assays include calcium-imaging of the ligand affinity of receptors expressed in human embryonic kidney cells (HEK) (Corcoran *et al.*, 2014; Große-Wilde *et al.*, 2006; Syed *et al.*, 2006) and cell cultures of *Spodoptera frugiperda* (Sf9) (Xu *et al.*, 2015; Anderson *et al.*, 2009; Jordan *et al.*, 2009; Kiely *et al.*, 2007; Matarazzo *et al.*, 2005) or electrophysiological, patch clamp recordings of receptors expressed in the membranes of *Xenopus* oocytes (Jiang *et al.*, 2014; Liu *et al.*, 2013; Zhang & Löfstedt, 2013; Leary *et al.*, 2012; Wanner *et al.*, 2010a; Mitsuno *et al.*, 2008; Sakurai *et al.*, 2004). Mutant *D. melanogaster* fly lines have been used for *in vivo* expression and deorphanization. Here, one of the endogenous receptors is impaired and instead the OSNs transgenically express the OR to be deorphanized through single sensillum recordings assays (SSRs) (Pellegrino *et al.*, 2010; Ignell & Hansson, 2005; Hallem *et al.*, 2004; Dobritsa *et al.*, 2003; Hansson, 1995).

Gustatory receptors (GRs)

GRs are proteins that mediate contact chemosensation (Chapman, 2003). They are phylogenetically more ancient than insect ORs, and it is considered that olfactory genes have in fact evolved from GRs genes (Engsontia *et al.*, 2014; Missbach *et al.*, 2014; Hallem *et al.*, 2006). Just like ORs, they contain seven transmembrane domains (Agnihotri *et al.*, 2016). Multiple types of GRs are expressed in the membranes of chemosensory neurons housed in uniporous sensilla present on mouthparts, wings, legs, ovipositor and internal structures of the pharynx (Benton, 2015; Depetris-Chauvin *et al.*, 2015; van Loon *et al.*, 2005). In addition, some GRs are present in adult olfactory organs (Fujii *et al.*, 2015).

The main function of insect GRs is to determine the quality of food sources, a primary reason why these receptors are considered determinants of host acceptance (van Loon *et al.*, 2005). Sugars, amino acids, salts and minor minerals are among the nutrients detected by GRs (Chapman, 2003). A second

function of GRs is the detection of secondary metabolites that act as phagostimulants or as deterrents. Most of these GRs are tuned to non-volatile compounds, but there are also GRs tuned to CO_2 (Jones *et al.*, 2007). Finally, studies with *D. melanogaster* indicate that some GRs also influence courtship by tasting non-volatile pheromones (Everaerts *et al.*, 2010; Moon *et al.*, 2009; Villella & Hall, 2008).

Elucidation of the specific functions of insect GRs have been carried out for a handful of receptors of the fruit fly, mosquitoes and a few moth species. For this purpose, several studies have used RNA interference (RNAi), heterologous expression in *Xenopus* oocytes and traditional SSRs from gustatory organs (Freeman & Dahanukar, 2015; Erdelyan *et al.*, 2012; Sato *et al.*, 2011). These functional studies indicate that, regardless of the divergence of this gene family, there are phylogenetically defined clusters of GRs that respond to sugars, CO_2 and bitter compounds (Freeman & Dahanukar, 2015; Miyamoto *et al.*, 2012; Weiss *et al.*, 2011; Moon *et al.*, 2009; Clyne *et al.*, 2000).

The number and functions of GRs present in each insect species are variable but linked to their ecological needs (van Loon *et al.*, 2005). Increasing evidence is showing how the evolution of GRs might be involved in the adaption towards new ecological niches or host shifts (Agnihotri *et al.*, 2016; Engsontia *et al.*, 2014). For example, it has been suggested that development of clade expansions of genes encoding GRs tuned to bitter compounds might have favoured polyphagy in heliothine moths (Xu *et al.*, 2016) and in the pest *Drosophila suzukii* (Crava et al., 2016).

Ionotropic receptors (IRs)

IRs constitute the second "nose" in insects. IRs sense, among other functions, distinct classes of odourants that ORs do not typically detect (Silbering *et al.*, 2011; Croset *et al.*, 2010). IRs, diverge from ionotropic glutamate receptors (iGluRs), an independent and more ancient gene family than GRs and ORs (Missbach *et al.*, 2014; Benton, 2009).

In *D. melanogaster*, IRs are expressed in sensilla coeloconica and in other sensory structures called arista and the sacculus (Rytz *et al.*, 2013). Electrophysiological recordings from these sensilla demonstrate their tuning towards amines, carboxylic acids and aldehydes, along with a role in thermo and hygrosensation (Hussain *et al.*, 2016; Knecht *et al.*, 2016; Silbering *et al.*, 2016; Silbering *et al.*, 2011).

Similar to ORs, some IRs appear to function as co-receptors of other IRs (IR8a, IR25a and IR76b), putatively forming heteromeric complexes that determine ligand specificity and sensitivity, but their specific mechanisms have yet to be determined (Rytz *et al.*, 2013; Abuin *et al.*, 2011).

IRs have been comparatively less studied than insect ORs. However, the presence of close orthologs of characterized *D. melanogaster* IRs has been predicted in mosquitoes, bees and moths (Rytz *et al.*, 2013). Although functional characterization has been widely performed in the fruit fly, predicted conserved functions in other species need to be demonstrated.

Sensory neuron membrane proteins (SNMPs)

SNMPs are related to the CD36 gene family widely conserved in multiple vertebrate and invertebrate animals (Nichols & Vogt, 2008; Benton *et al.*, 2007). This gene family produces membrane-embedded glycoproteins that recognize and transport lipoproteins, fatty acids and oxidized phospholipids into the cell (Stengl, 2010; Silverstein & Febbraio, 2009).

Two main subfamilies of SNMPs have been found, SNMP1 and SNMP2 (Nichols & Vogt, 2008). Low conservation and differential location and expression of both families have been observed in moths and locusts: SNMP1 is expressed in pheromone sensing neurons whereas SNMP2 is expressed in auxiliary cells surrounding the OSNs (Jiang *et al.*, 2016; Forstner *et al.*, 2008). Studies with mutant *Drosophila* flies with non-functional SNMPs showed that in T1 sensilla lacking SNMPs, the characteristic response to the pheromone cis-vaccenyl acetate (cVA), was completely abolished, demonstrating that these proteins are required for the proper detection of pheromones (Benton *et al.*, 2007). Similar results have been observed in pheromone reception of heliothine moths, in which the co-expression of SNMPs and PRs increased the response towards pheromone stimulation by up to 1000 times (Pregitzer *et al.*, 2014).

Recent studies suggest that SNMPs expressed in OSNs might lower the energy barrier of pheromones to associate and dissociate from PRs (Li *et al.*, 2014). Furthermore, structural *in vivo* tests show that the ectodomain of SNMP1 plays the most important part in accommodating and funnelling pheromones from the sensillar lymph towards the relevant PRs (Gomez-Diaz *et al.*, 2016). SNMP2 functions seem less clear, however a putative role in discarding obsolete odours has been suggested (Huang *et al.*, 2016a).

Odourant Binding Proteins (OBPs)

Insect OBPs belong to a superfamily of small acidic water-soluble carrier proteins, characterized by containing six α-helical domains with six highly conserved cysteines (Sun *et al.*, 2016; Pelosi *et al.*, 2014; Sandler *et al.*, 2000; Leal *et al.*, 1999). Typically, OBPs are the most highly abundant proteins expressed in peripheral organs, specifically released in the sensillar lymph by

the auxiliary cells (tormogen and trichogen) in high densities (Sun *et al.*, 2016; Suh *et al.*, 2014).

It is generally considered that the main function of OBPs is the solubilisation and transportation of hydrophobic odours across the sensillar lymph. In addition, pheromone-binding proteins (PBPs) are thought to participate in pheromone selectivity and sensitivity (Leal, 2013; Ha & Smith, 2006; Xu *et al.*, 2005; Wojtasek & Leal, 1999). The most recognized example of PBP-pheromone association is the case of the male-produced *D. melanogaster* pheromone, cVA, and the PBP *lush* (Billeter & Levine, 2015). *Lush* is expressed in T1 sensilla and carries the pheromone cVA; hence mutations of this protein are reflected in irregular activity of the neuron containing the cVA receptor (OR67d) and disruption of the courtship and aggregation behaviours associated with this pheromone (Ronderos & Smith, 2010; Ha & Smith, 2006; Xu *et al.*, 2005). Furthermore, *lush* seems to have a putative role in perception of other pheromones in addition to cVA (Billeter & Levine, 2015).

The specific mechanism of OBP functioning is still a matter of controversy. However, biochemical and structural studies of PBPs indicate that PRs may be activated not by the pheromonal compound itself but by the complex of PBP and pheromone (Leal, 2013). Wojtasek and Leal (1999) showed that PBP1 of *Bombyx mori* binds with the pheromone bombykol at sensillar pH, but disassociates at lower pH, such as the one of OSNs membranes. Therefore, it is possible that PBPs release pheromones to their corresponding PRs by conformational changes due to the acidity of the membranes in which the receptors are embedded (Leal, 2013). Furthermore, the complexity of OBP functionality has been extended in a recent study, showing that certain types of OBPs are associated with specific types of sensilla and that their functions are not only or limited to odourant transportation (Larter *et al.*, 2016). By mapping the gene expression of OBPs in *Drosophila* antennae, Larter et al. (2016), discovered a single OBP (Obp28a) with abundant expression in a particular type of sensilla basiconic, evidenced by the mutant line, this protein was not necessary for odourant detection. Instead, it seems Obp28a acts as a gain control mechanism that buffers sudden changes in odour concentration.

Odourant degrading enzymes (ODEs)

Contrary to other chemosensory-related proteins, ODEs are a heterogeneous group of enzymes belonging to different gene families, including esterases, aldehyde oxidases, cytochromes P450s and glutathione S-transferases (Merlin *et al.*, 2005). Nevertheless, high antennal abundance of esterases and their putative role in degrading pheromones has been demonstrated in several

insects such as *D. melanogaster* (Chertemps et al., 2015), *Spodoptera littoralis* (Durand et al., 2011), *Bombyx mori* (Yu et al., 2009), *Mamestra brassicae* (Maïbèche-Coisne et al., 2004) and *Antheraea polyphemus* (Ishida & Leal, 2002).

The function of ODEs is to terminate the receptor response by degrading chemical signals. This allows insects to better locate the position of the odour source during navigation. However, the mechanism still needs to be further elucidated. To date, most *in vitro* data and emerging *in vivo* studies point towards a rapid degradation of odourants by antennal ODEs, particularly esterases (Chertemps *et al.*, 2015; Chertemps *et al.*, 2012). However, the possibility of other molecular actors participating in odourant deactivation cannot be ruled out (Rützler & Zwiebel, 2005).

2.1.3 Insect Olfactory Pathway

OBPs, SNMPs, ORs and ODEs contribute to the perception of environmental odours, but all of this machinery is housed inside the sensilla on the antenna and maxillary palps (Leal, 2013). To cause a behavioural effect, the interaction of the right receptor with the right odourant must lead from a transduction cascade to higher brain centres (Figure 2).

Subsequent to the receptor-odourant interaction at the periphery, action potentials generated at the soma of OSNs are transmitted to the antennal lobe (AL), the primary olfactory centre in the insect brain (Reisenman & Riffell, 2015; Galizia & Lledo, 2013; Masse et al., 2009). The AL is constituted of a species-specific number of globular neuropil, called glomeruli, in which (in most of cases) axons of neurons expressing the same type of receptors converge in the same glomerulus (Gadenne et al., 2016; Anton & Homberg, 1999).

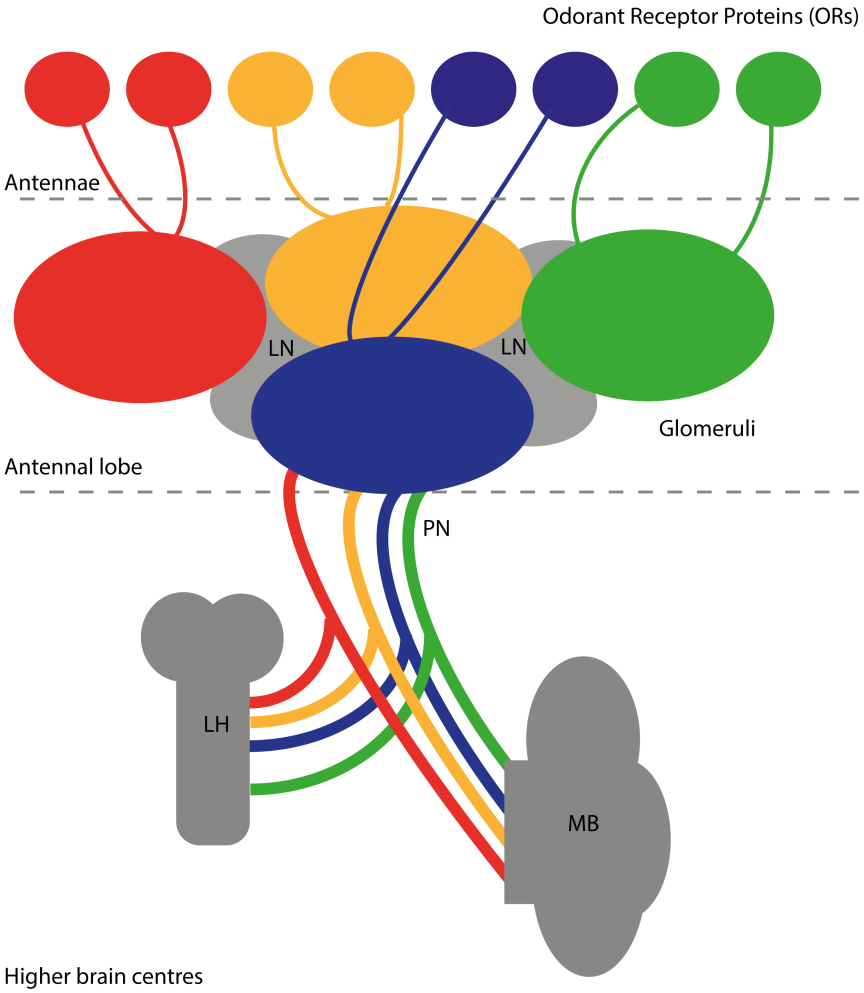

Figure 2. Schematic representation of insect olfaction at different olfactory levels. LN, PN, LH and MB stand for local interneurons, projection neurons, lateral horn and mushroom bodies, respectively. Different colours indicate different OR types.

The signal fed from the peripheral OSNs into the AL is then processed through synaptic interactions between the axons of local interneurons that interconnect different glomeruli, and exits through projection neurons towards higher brain centres (Depetris-Chauvin *et al.*, 2015). These brain centres are the lateral horn of the protocerebrum and the calyces of the mushroom bodies. Signals integrated in the mushroom bodies (MB) are related to decision-making, learning and memory (Heisenberg, 2003; Zars, 2000). On the other hand, signals integrated at the lateral horn (LH) are hypothesized to be involved in innate and instinctive behaviour (Roussel *et al.*, 2014; Gupta & Stopfer, 2012).

2.1.4 Evolution of Insect Chemoreceptors

As mentioned above, IRs are the most ancient type of olfactory receptors in insects, and are present in all Protostomes (Abuin *et al.*, 2011; Croset *et al.*, 2010). However, insect ORs and GRs have a different common origin, possibly traced back to the adaptation of marine organisms to terrestrial lifestyles, with a possible expansion of these gene families as an evolutionary response to the diversification of vascular plants and coincident with the development of flight in insects (Missbach *et al.*, 2014).

The insect chemosensory receptor superfamily, comprised of ORs and GRs, evolves through the typical birth-and-death process (Nei & Rooney, 2005). This means that olfactory genes arise from tandem duplications, i.e. from one gene a derived gene is produced (Sanchez-Gracia *et al.*, 2009). These new genes then follow several possible fates. If the new gene is functionally redundant, one of the copies may suffer a deleterious or inactivating mutation and therefore be lost (pseudogenization). If the new gene is functional and embraces mutations that allow the detection of new and ecologically relevant odourants, the gene would be under positive selection, diverge from the original and be fixed as a new olfactory gene. When mutations compromise the functioning and tuning of ORs, the genes are under negative (purifying) selection, i.e., evolution prevents non-synonymous substitutions to maintain the original function (Ache & Young, 2005). These dynamics are usually measured through the ratio of substitutions at non-synonymous (dn) and synonymous sites (ds), with the general assumption that dn/ds>1 is an indicator of positive selection acting as evolutionary pressure of divergent proteins (Nei & Kumar, 2000).

The processes of rapid gene gains and losses in olfactory genes have allowed the rise of species-specific expansions and contractions. Agonism of new receptors and ligands can lead to changes in behavioural preferences and therefore contribute to host shifts and preference towards novel semiochemicals, leading to speciation through means of divergent natural and sexual selection (Smadja & Butlin, 2009; Rundle & Nosil, 2005).

2.1.5 Role of Chemoreceptors in Speciation

The evolution of chemosensory systems provides an excellent source of information on the role of chemosensation in speciation. New species arise mainly from two mechanisms: mutation-speciation in which populations under similar environments are separated by the rise and fixation of contrasting mutations and ecological speciation when divergence is promoted by contrasting environments (Schluter, 2009). Mutation speciation is observed with pheromone production and pheromone detection genes (Linn & Roefols, 1995). However, in a broader sense the evolution of chemosensation in insects also fits the concept of ecological speciation since host choice is directly correlated with mate choice, i.e. insects will prefer hosts that increases their chances to encounter sexual partners that also prefer those hosts and that will allow more sexual opportunities for their offspring (Smadja & Butlin, 2009; Quental *et al.*, 2007; Rundle & Nosil, 2005). Such preferences lead to patterns of assortative mating and therefore reproductive isolation, which has been often considered as a key component of sympatric speciation (Smadja & Butlin, 2009; Berlocher & Feder, 2002; Drès & Mallet, 2002). Increasing number of studies support the role of host preferences as driving forces of reproductive isolation (Malausa *et al.*, 2005; Emelianov *et al.*, 2003; Linn *et al.*, 2003).

One of the best-documented cases of olfactory-mediated speciation is found in *Drosophila* flies. OR responsiveness tests have demonstrated that ubiquitous fruit flies with a broad diet use a set ORs that detect microbial and plant volatiles, whereas in flies with narrow host spectra such as *D. sechellia, D. mojavensis* and *D. erecta*, ORs are tuned to characteristic odours of their hosts, morinda, cacti and screw pine fruit, respectively (Crowley-Gall *et al.*, 2016; Linz *et al.*, 2013; Stensmyr, 2009; McBride, 2007; Dekker *et al.*, 2006; R'kha *et al.*, 1991). Furthermore, evolutionary studies have shown that for *D. sechellia* and *D. erecta,* host specialization also correlates with higher rates of amino acid substitutions and gene loss, supporting the notion that adaptation to new hosts mirrors changes in the odourant receptor repertoires (McBride & Arguello, 2007). Moreover, a difference in a single amino acid in IR75b of *D. sechellia* has been pointed out as responsible for the sensitivity and attraction of this species towards hexanoic acid, a key host cue for this species, but not for *D. melanogaster* (Prieto-Godino *et al.*, 2017). Recently, a new study shows how the OR repertoire of *D. suzukii* has evolved through positive selection towards ORs responding to short-chain esters, parallel to the loss of ORs tuned to fermentation products. These mutations correlate with the ecological needs of *D. suzukii* which has a preference for ripening fruits (which produce an abundance of esters) rather than overripe fruits (which produce an abundance

of yeast-derived VOCs) as preferred by other drosophilids (Ramasamy *et al.*, 2016).

2.2 Insects Studied

2.2.1 Cydia pomonella

Codling moth, *Cydia pomonella* (L.), is the most important pest of apple worldwide. Additionally, the larvae feed in pear, walnut and other tree fruit (Shorey & Gerber, 1996; Howell *et al.*, 1992). Increasing cases of pesticide resistance and shorter life cycles due to climate change make this species a main target for pest control (Stoeckli *et al.*, 2012; Reyes *et al.*, 2007). Since the elucidation of its main pheromone component (*E,E*)-8,10-dodecadienol (codlemone), this species has emerged as a classic model of pest control through mating disruption, i.e., a pest management strategy based on air permeation with synthetic pheromone that impedes males from finding sexually receptive females (Witzgall *et al.*, 2008).

Codlemone is produced in the pheromone glands of codling moth females from palmitic acid by two cycles of β-oxidations and a *E*9 desaturation of dodecanoic acid followed by a transformation of the *E*9 double bond into the (*E,E*)-8,10-diene, with a final reduction of the acid into an alcohol, which represents a characteristic feature of this species (Löfstedt & Bengtsson, 1988). Many other species of the same family of the codling moth (Tortricidae) use different combinations of isomers of (Δ,Δ)-8,10-dodecadienyl acetate (codlemone acetate) in their pheromone blends (Witzgall *et al.*, 2010b). Interestingly, in the case of codling moth, codlemone acetate and other isomers of codlemone, act as a pheromone antagonists, i.e. compounds that promote reproductive isolation between species that share common habitats and pheromonal compounds (Juárez *et al.*, 2016; Gemeno *et al.*, 2006; Witzgall *et al.*, 2001; Baker, 1997; Vickers & Baker, 1997).

Despite the apparent success of mating disruption for codling moth control, there are many cases in which this method does not suffice to keep populations under economical thresholds. The main reason is that mating disruption with codlemone targets only males, rather than females and larvae, precluding mating disruption success in cases of high infestations and migrations of gravid females (Witzgall *et al.*, 2010a; Witzgall *et al.*, 2008). Therefore, search for environmental cues that might attract females and neonates has been carried out in parallel to pheromone research.

Several VOCs of plant and microbe origin have shown antennal activity, for example (*E,E*)-α-farnesene, linalool, germacrene-D, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (Z)-3-hexenol, (Z)-3-hexenyl hexanoate, butyl hexanoate,

butyl acetate and isoamyl acetate (Witzgall *et al.*, 2012; Ansebo *et al.*, 2004; Hern & Dorn, 2004). However, the most powerful attractant identified so far is a pear-derived kairomone, ethyl-(*E,Z*)-2,4-decadienoate (pear ester), which elicits antennal responses and chemotaxis in males, females and larvae of codling moth (Light & Beck, 2010; Knight & Light, 2001; Light *et al.*, 2001). Furthermore, the importance of pear ester is underscored by the demonstration of its role as pheromone synergist (Trona *et al.*, 2010a; Trona *et al.*, 2010b; Knight & Light, 2005).

SSRs from codling moth antennae have indicated the existence of channels for the detection of codlemone, codlemone acetate and its isomers, as well as pear ester (Ansebo *et al.*, 2005; Bäckman *et al.*, 2000). However, these recordings do not allow full comprehension of the molecular basis of detection of these semiochemicals. As a first step towards this goal, an antennal transcriptomic study was carried out to determine the olfactory and gustatory receptor repertoires of codling moth (Bengtsson *et al.*, 2012). In this study a total of 43 ORs, 15 IRs and one GR were predicted, which in general represents lower numbers than what has been consistently observed in other moths such as *Bombyx mori*, *Helicoverpa assulta* and *Epiphyas postvittana* (Corcoran *et al.*, 2015; Xu *et al.*, 2015; Wanner & Robertson, 2008). Nevertheless, this study established the basis for deorphanization of codling moth chemoreceptors.

2.2.2 Drosophila melanogaster

Insect chemosensory studies have been facilitated by the use of the model organism, *D. melanogaster*. Knowledge derived from genomic studies has enabled the production of mutants to test multiple olfaction-related questions. In addition, simple behavioural tests and relatively fast life cycles, have established *Drosophila* as a model in insect olfaction research (Couto *et al.*, 2005; de Bruijne, 2003; Warr *et al.*, 2001; Stocker, 1994).

A total of 60 genes encoding for 62 ORs have been predicted in *D. melanogaster* (Clyne et al., 1999; Gao & Chess, 1999; Vosshall et al., 1999). Deorphanization of most of these receptors has been achieved, revealing their responses towards approximately 700 odourants of diverse origin, including plant-derived, microbe-associated and fly-produced odours (Münch & Galizia, 2016; Galizia *et al.*, 2010; van Naters & Carlson, 2007; Hallem & Carlson, 2006; Hallem *et al.*, 2006).

Many of the deorphanized receptors respond to VOCs produced by yeasts or other indicators of decaying fruit, which coincide with the niche of these flies, since they aggregate, feed, mate and develop on these substrates (Becher

et al., 2012; Lebreton *et al.*, 2012; Becher *et al.*, 2010). In the specific case of oviposition, fruit flies prefer to lay eggs in yeasts growing on citrus fruit. Female flies possess a single dedicated olfactory channel to detect the terpenes associated to these substrates (Dweck *et al.*, 2013). In addition, a dedicated receptor has been found for the detection of the compound geosmin (an indicator of harmful pathogen growth), exemplifying the importance of ORs to discriminate between good and potentially unsafe hosts (Stensmyr *et al.*, 2012).

Sexual communication in the fruit fly has been widely studied (Billeter & Levine, 2015; Laturney & Billeter, 2013; Ferveur, 2005; Sturtevant, 1915). However, the full picture is still far from complete. This is partly because the courtship behaviour of *Drosophila* flies involves a complex series of steps, mediated by multiple sensorial modalities (Figure 3).

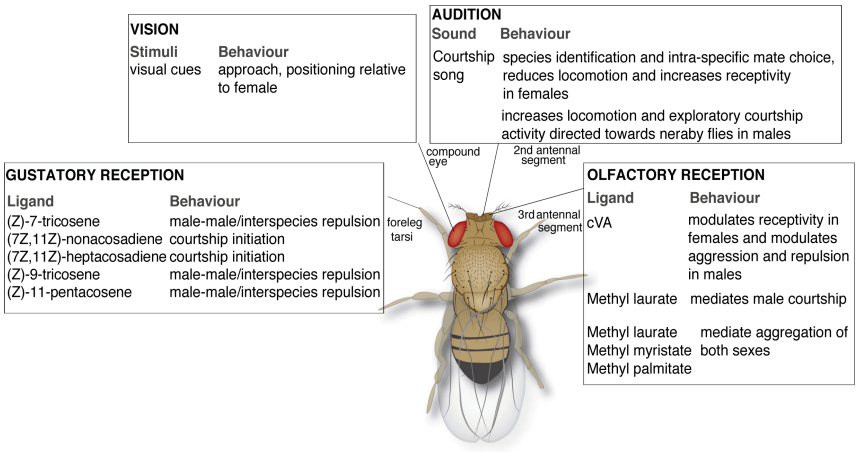

Figure 3. Examples of multisensorial control of social behaviours of *Drosophila melanogaster*. Modified from Auer & Benton (2016).

In the fruit fly, chemically-guided behaviours towards conspecifics are determined by both gustatory and olfactory receptors (Auer & Benton, 2016; Gaudry *et al.*, 2012; Gomez-Marin & Louis, 2012; Everaerts *et al.*, 2010).

The pheromonal compounds of *Drosophila* can be divided into two groups: pheromones produced in oenocytes (specialized cells under the cuticle of the abdomen) and non-oenocyte derived pheromones. Oenocyte-produced pheromones are cuticular hydrocarbons (CHCs) that modulate mating and aggregation behaviours. CHCs indicate sexual and species identity (Billeter & Levine, 2015). For example, (*Z,Z*)-7,11-heptacosadiene and (*Z,Z*)-7,11-nanocosadiene act as female aphrodisiacs, whereas (*Z*)-7-tricosene is a male

antiaphrodisiac and promotes male to male aggression (Billeter & Levine, 2015; Greenspan & Ferveur, 2000). CHCs are contact pheromones, hence mediated through gustatory receptors (Everaerts *et al.*, 2010). In the group of non-oenocyte produced pheromones, the best known is the volatile cVA produced by the ejaculatory bulb of the male reproductive system. cVA increases female receptivity, inhibits male to male courtship, induces male-male aggression, decreases the attractiveness of mated females to other males and acts as a aggregation pheromone (Greenspan & Ferveur, 2000). Detection of cVA is mediated by OR67d receptor, expressed in OSNs housed inside of a particular type of trichoid sensilla, called T1 (Kurtovic *et al.*, 2007; Couto *et al.*, 2005). A second OR, OR65a, is responsible for the deactivation of attraction of females towards cVA once they have already mated, and also mediates a decrease in the aggression of mated males towards each other (Lebreton *et al.*, 2014; Liu *et al.*, 2011). Recently, three oenocyte-independent fly produced odours, namely methyl laurate, methyl myristate and methyl palmitate, have been reported as aggregation and sexual modulation pheromones, and the receptors OR47b and OR88a have been identified as responsible for their detection (Dweck *et al.*, 2015).

2.3 Current Questions in Insect Olfaction

Despite decades of research, our knowledge of insect olfaction is yet incomplete. At the level of the Orco-ORX-ligand interaction, the stoichiometry, mechanistics of complex formation and signal transduction are not well-understood (Hopf et al., 2015). One main reason is the lack of three-dimensional structure models, which is difficult to achieve with transmembrane proteins (Carraher et al., 2013). Nevertheless, computational models and direct mutagenesis have allowed for hypotheses on OR structural functioning (Hopf et al., 2015). Although several transmembrane and ectopic domains have been suggested as necessary for OR specificity, functional studies indicate that even non-conservative substitutions in the C-terminus can influence OR tuning (Hill *et al.*, 2015). Therefore, functional analysis of OR tuning between orthologs and structurally related chemical substances are required, since they can provide insights on the specific protein regions involved in the detection of insect semiochemicals.

A derived question regarding functional studies concerns the methods to functionally characterize insect ORs. In an attempt to answer this query, Wang *et al.* (2016) compared the most commonly used *in vitro* and *in vivo* methods, determining that whereas cell-based methods are suitable to test large numbers

of ORs, the *in vivo* system of transgenic *Drosophila* may produce better results pertaining to OR functioning. Intriguingly, just a handful of studies have used *Drosophila* heterologous expression to deorphanize insect ORs, suggesting a need for reviewing the available protocols.

With respect to the insects studied in this thesis, there are several questions regarding olfaction that need to be addressed. In *D. melanogaster*, a long-range female-produced pheromone, conveying species specificity, has not been found (Billeter & Levine, 2015). Furthermore, even while the tuning of fruit fly ORs has been widely studied, an intriguing question concerns the function and tuning of alternatively spliced products of insect olfactory genes (Garczynski & Leal, 2015). In the case of *Cydia pomonella*, some olfaction-related proteins such as OBPs, ODEs and SNMPs have been functionally characterized to some extent (Huang *et al.*, 2016a; Huang *et al.*, 2016b; Tian *et al.*, 2016; Tian & Zhang, 2016). However, ORs, the key proteins in olfaction, still need to be deorphanized in this species, particularly PRs. Bengtsson *et al*. (2012), identified a total of five putative PRs, which is interesting in view of six pheromone components that are known to elicit responses from at least four classes of OSNs of codling moth (Trona *et al.*, 2010a; Ansebo *et al.*, 2005; Witzgall *et al.*, 2001; Arn *et al.*, 1985).

One of the most intriguing questions in insect olfaction is how detection of pheromones and kairomones, the smells of sociality, has evolved. Insects detect pheromones and host odours together, which means that both natural and sexual selection are acting on the genes mediating the detection of such chemical signals (Safran *et al.*, 2013; Servedio *et al.*, 2011; Smadja & Butlin, 2009). Habitat cues and pheromones interact in nature. Two examples that epitomize this principle are the higher attraction of *D. melanogaster* females to food sources in which cVA is simultaneously released (Laturney & Billeter, 2013), and low attraction of *Spodoptera littoralis* to pheromones when the background presents herbivore-induced VOCs (Hatano *et al.*, 2015).

Physiological consequences of the interaction between pheromones and habitat cues at the peripheral level vary: co-application of pheromones and plant compounds may increase or decrease the firing activity of OSNs depending on the species and compounds tested (Ammagarahalli & Gemeno, 2015; Hatano *et al.*, 2015; Andersson *et al.*, 2010; Party *et al.*, 2009; Ochieng *et al.*, 2002). Plant compounds that suppress pheromonal compounds may indicate non-hosts, or low quality plants. Detection of both types of signals simultaneously represents an advantage for insects in terms of spatiotemporal resolution, i.e. an increase in specificity and sensitivity and decrease in time of response (Andersson, 2012; Baker, 2009; Krieger *et al.*, 2009). This is exemplified by the co-localization of pheromone and host signals detecting

OSNs in the same sensillum in bark beetles (Andersson *et al.*, 2010). Further studies are needed to determine whether pheromones and synergists may interact at the periphery in a similar way.

Finally, an outstanding question relates to how to use the knowledge of chemoreceptors to control other insect species that threaten human activities. Taking into account that the family Tortricidae contains many agricultural pests, studying and deciphering the evolution of ORs in codling moth may provide new information on the role of different semiochemicals in the behavioural ecology of tortricids. Similarly, examining the evolution of receptor tuning in the fruit fly, *Drosophila melanogaster,* can also provide insights on another drosophilid of utmost agricultural importance, the spotted wing *Drosophila* (*D. suzukii*).

3 Aim and Objectives

The general goal of this thesis was to increase our knowledge of how evolution has shaped the detection of social and environmental cues in insects.

The first part of this thesis provides a closer look at the chemoreceptors of codling moth, *Cydia pomonella,* and the functional characterization of its odourant receptors. The specific objectives were:

- Provide a more complete and comprehensive identification and quantification of codling moth chemoreceptors expressed in adult males, females and larvae (Paper I).
- Functionally characterize key candidate pheromone receptors (Paper II and III).
- Gain insights on evolution of responsiveness and the role of chemical structures of ligands in OR tuning (Paper IV)
- Provide a step-by-step description of our methodology for functional characterization of insect ORs (Paper V).

The second part of this thesis investigates the ecological significance and implications of ORs that interplay social and environmental cues in codling moth and in the fruit fly, *Drosophila melanogaster*. The specific goals were:

- Corroborate the presence of the kairomone pear ester in apple orchards and its role in host finding (Paper VI).
- Determine the function of two alternatively spliced variants of a receptor in perception of social and host related cues in *D. melanogaster* (Paper VII).

Finally, the third part of this thesis relates functionally characterized receptors of codling moth with putative conserved functions in other tortricids of economical importance. The specific objective was:

- Provide a transcriptomic identification and quantification of chemoreceptors putatively tuned to social and environmental cues of three tortricid moths that are phylogenetically close to codling moth (Paper VIII).

4 Summary of Results and Discussion

4.1 Part 1: Functional Characterization of Codling Moth ORs

Through an RNA-Seq approach we provided an update and revision of the chemosensory gene families of codling moth reported earlier by Bengtsson *et al.* (2012). By extracting RNA from adult male and female antennae and from neonate larval heads, we extended previously predicted sets of ORs, GRs and IRs. Furthermore, we provided transcript abundance estimations for the predicted chemosensory receptors. This comparison allowed us to determine sexually biased and larval enriched receptors (Figure 4). These results highlighted the potential importance of the most highly expressed putative PRs, CpomOR1 and CpomOR6 in males and CpomOR3 in females, and also suggest several new candidates ORs for future functional studies (Chapter I).

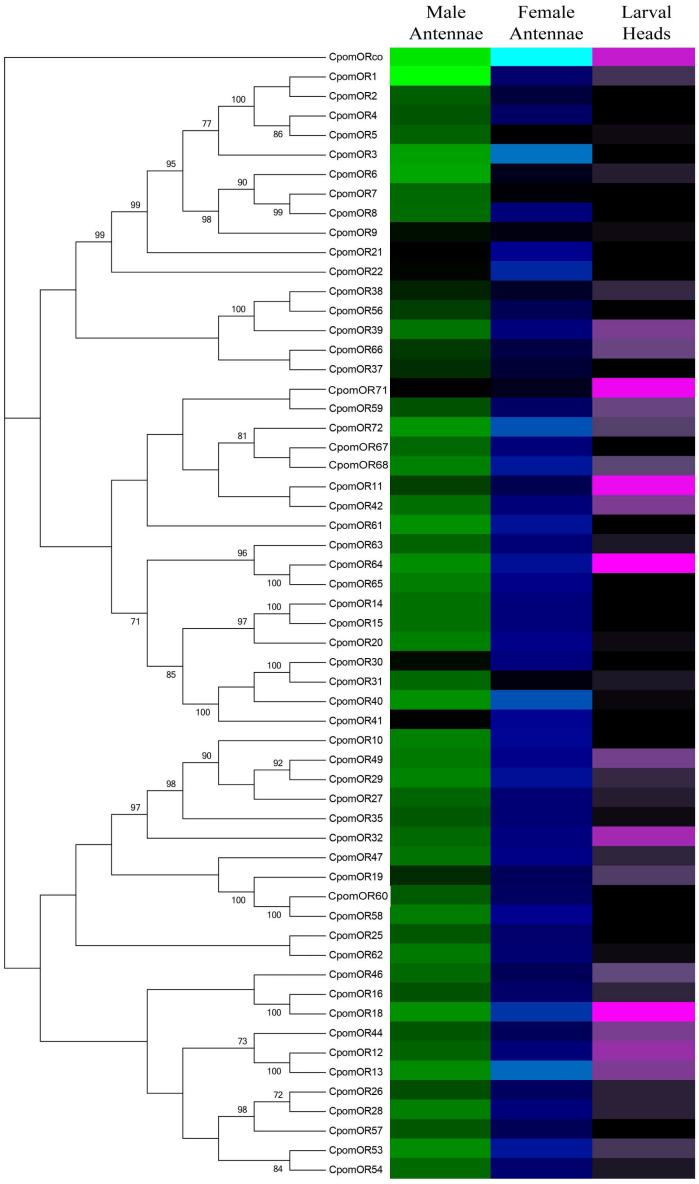

Figure 4. Phylogeny of codling moth odourant receptors and heat-plots of relative expression values determined by read mapping. Black indicates low/no expression, dark colors indicate low/moderate expression, and bright colors indicate moderate/high expression. Relative expression levels are relevant within, but not across columns.

Through heterologous expression of predicted PRs of codling moth, we functionally characterized the receptors CpomOR3 and CpomOR6. Although originally predicted to be a pheromone receptor, CpomOR3 is tuned to pear ester, one of the most important kairomones for codling moth. Transcript abundance estimations observed in males and females, in addition to both the specificity and sensitivity of CpomOR3 towards pear ester (Figure 5), underscores the importance of this receptor for pear ester detection (Chapter II).

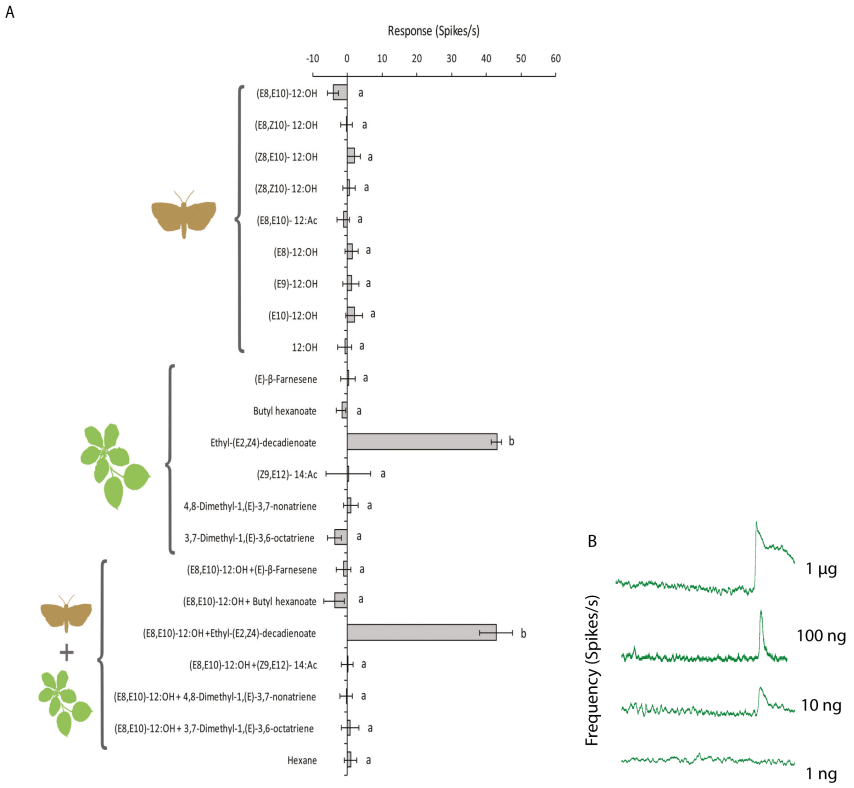

Figure 5. Response of CpomOR3 transgenically expressed in *Drosophila melanogaster*. A) Response of CpomOR3 towards pheromones and kairomones associated to codling moth. B) Dose-dependent response of CpomOR3 towards pear ester evaluated with single sensillum recordings coupled with gas chromatography (GC-SSR).

CpomOR6a responds to codlemone acetate, its geometric isomers and to the compound (*E*)-10-dodecenyl acetate (Figure 6). These results confirm the prediction by Bäckman *et al*. (2000), of the existence of a dedicated neuron with receptors tuned to codlemone acetate and isomers, which is entirely independent from codlemone detection (Chapter III).

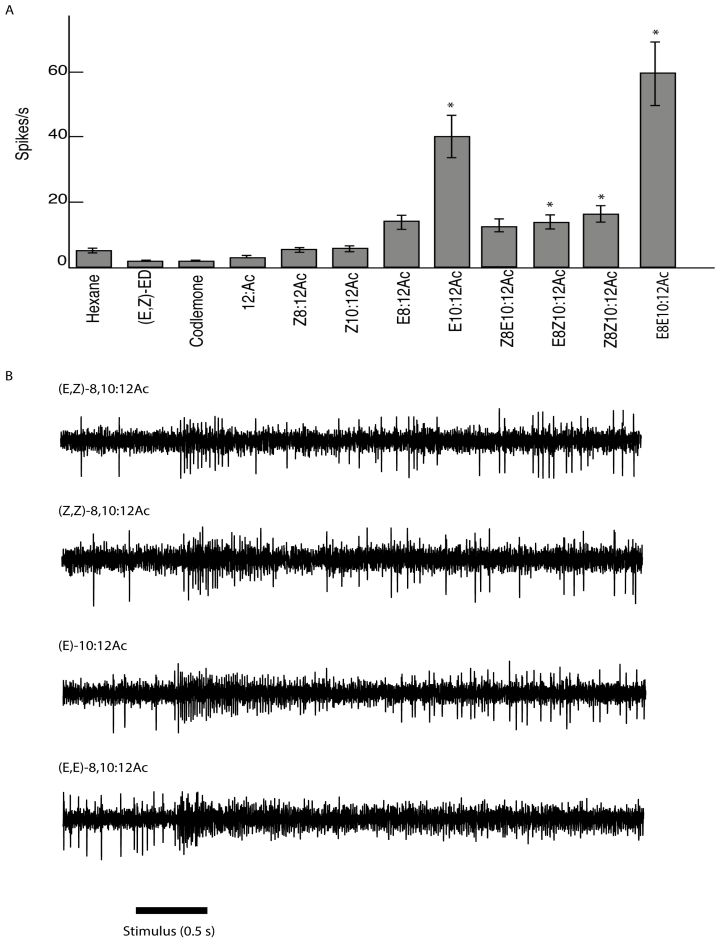

Figure 6. Response of CpomOR6a towards different codling moth pheromone components, synergists and antagonists when transgenically expressed in *Drosophila melanogaster*. A) Average response of CpomOR6a. Asterisks indicate significant differences between the compound and the solvent (Mann Whitney-U test, $P < 0.05$, n =9). B) Spike trains from basiconic sensilla ab3 expressing CpomOR6a.

When it comes to codlemone, based on phylogenetic relationships and the transcript abundance estimation in male adult antennae, we predict CpomOR1 as its primary receptor. However, our attempts to deorphanize CpomOR1 were unsuccessful using two expression platforms, HEK293T cells and the empty neuron system in *D. melanogaster*. Codlemone detection may depend on the interaction of CpomOR1 with other co-factors such as PBPs and SNMP1, as observed with the pheromone cVA in *D. melanogaster* (Ronderos & Smith, 2010; Benton *et al.*, 2007; Ha & Smith, 2006; Xu *et al.*, 2005). CpomPBP1 was shown to have high affinity for codlemone via *in vitro* binding, while the role of CpomSNMP1 still needs further elucidation (Huang *et al.*, 2016a; Tian & Zhang, 2016).

Apart from deorphanization of PRs, we functionally characterized the receptor CpomOR19. Moreover, we compared the responsiveness of CpomOR19 and its ortholog in *Spodoptera litoralis*, SlitOR19, to gain insights on the effects of amino acid sequence differences between receptors, with relation to their ligand affinity. Our results indicate that, although both receptors share 58% amino acid identity and 69% amino acid similarity, their responsiveness is conserved. A tentative explanation is that most of point mutations concern regions that are probably not associated to ligand binding (Figure 7; Hopf *et al.*, 2015). In addition, by testing structurally related chemicals, we determined the chemical features of the ligands that elicited the strongest responses on the OR19 orthologs. These results bring us closer to identifying the natural ligands and therefore unveiling the evolutionary adaptation that this receptor represents (Chapter IV).

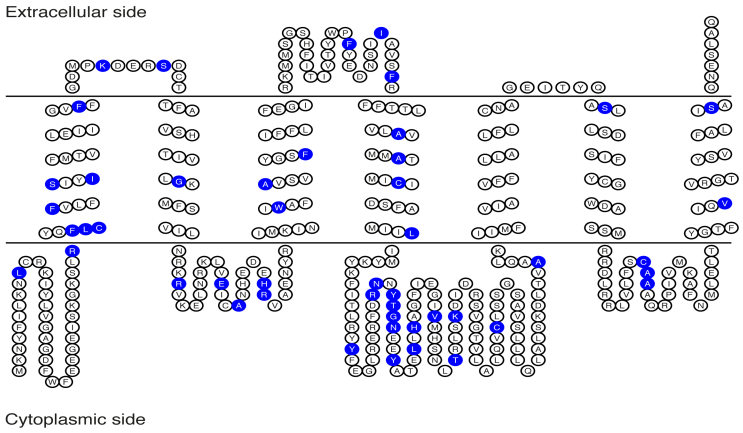

Figure 7. Putative transmembrane topology of CpomOR19. Residues in the colour blue represent non-conservative mutations compared to SlitOR19.

Taking advantage of the successful experiences deorphanizing codling moth ORs, we decided to revise and report a detailed account of the protocols used to generate transgenic flies expressing insect odourant receptors (Figure 8). We provided a step-by-step guide to manufacture testable flies either expressing ORs putatively tuned to general odourants through the so-called "empty-neuron system" using a OR22a-Gal4 line (Hallem *et al.*, 2004; Dobritsa *et al.*, 2003), or putative PRs through the use of knock-in mutant flies in which the OR67d receptor of Drosophila is replaced with an OR67d-Gal4 construct (Kurtovic *et al.*, 2007). In addition, we supply a troubleshooting guide for both, molecular and electrophysiological techniques (Chapter V).

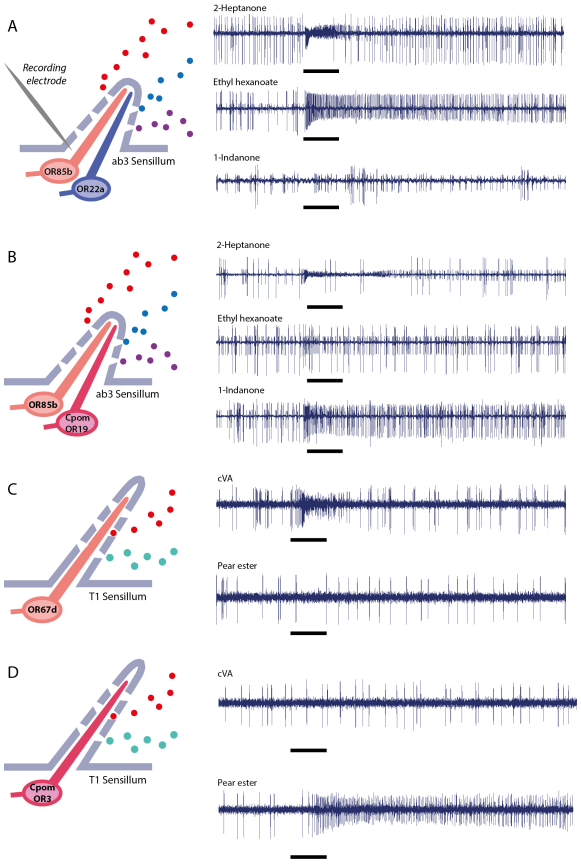

Figure 8. Example of heterologous expression of ORs and PRs in *Drosophila melanogaster*. In ab3 empty neuron system, (A) wild-type flies expressing native ORs, (B) mutant flies expressing native OR85b in the small neuron and transgenic CpomOR19 in the large neuron. In T1 knock-in mutant flies, (C) wild-type flies expressing native OR67d, (D) mutant flies expressing transgenic CpomOR3.

4.2 Part 2: Ecological Relevance of ORs in Social Interactions

When DNA sequences are derived from a common ancestor, their sequences diverge by the accumulation of nucleotide substitutions that may grant new functions to the proteins they code for (Nei & Kumar, 2000). CpomOR3 is phylogenetically close to *Cydia pomonella* PRs, yet it responds to a kairomone, rather than to a pheromone. Furthermore, our studies, demonstrated other important features of CpomOR3: the receptor is highly expressed in adult moths and it is quite sensitive to the detection of its ligand.

Until now, pear ester has been considered a pear-derived kairomone, even though the preferred host of codling moth is apple (Knight & Light, 2001). Taking into account the similarities of CpomOR3 with moth PRs, and considering the fact that this type of receptors can detect pheromones even when they are released in infinitesimal amounts, we hypothesized that the presence of pear ester in codling moth habitats, other than pears, might have been overlooked. By collecting volatiles from apples of two varieties during a whole season, we found that, just as predicted, pear ester is present in apples in increasing amounts during maturation (Figure 9). Interestingly, the amounts of pear ester are even lower than the amount of sex pheromone released by calling females, 5-7 ng/female/h (Bäckman *et al.*, 1997), which highlights the sensitivity of the olfactory pathway dedicated to this kairomone.

We propose a fundamental role of pear ester as a host-finding cue. The prevailing concept of host finding in chemical ecology is that insects find their host through perception of blends of ubiquitous compounds, rather than detecting species-specific plant volatiles (Bruce & Pickett, 2011). In contrast to this idea, our wind-tunnel results show that attraction of both sexes to synthetic pear ester, which is rather specific for apple and pear, is higher than to apple branches (Figure 10). Pear ester plays a role in social interactions, not only because it synergizes codlemone (Trona *et al.*, 2010a), but also because it indicates the proper host for females (Landolt *et al.*, 2007; Light & Knight, 2005). Therefore, pear ester also contributes to mate-finding in codling moth, as it is the case with a combination of yeast volatiles and cVA in *Drosophila* (El-Sayed *et al.*, 2013; Laturney & Billeter, 2013; Reddy & Guerrero, 2004). Tight association of pear ester and codling moth supports the "sensory drive hypothesis". This hypothesis states that evolution favours environmental signals that convey sexual information increasing the chances for mating (Endler, 1993; Endler, 1992). Our results demonstrate that pear ester is: a) a host cue that is released from apples, b) females and males recognize it above other olfactory cues and c) it is detected by an extremely sensitive and highly expressed receptor. These results are in accordance with the sensory drive hypothesis (Boughman, 2002).

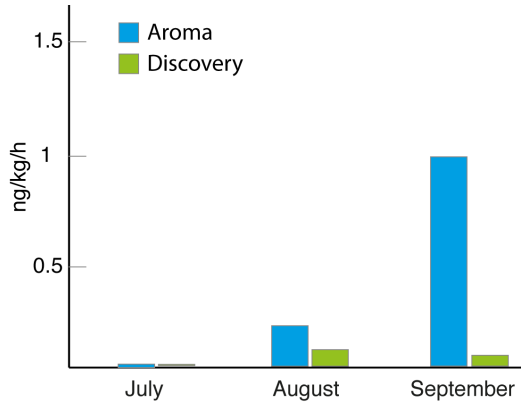

Figure 9. Abundance of pear ester in two varieties of apples sampled across the fruit-growing season.

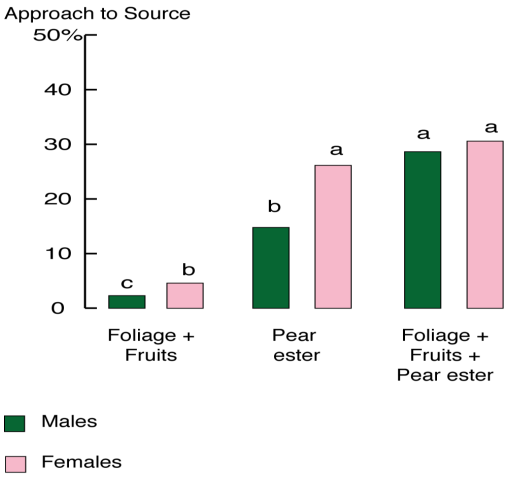

Figure 10. Flight response of codling moth males and females to pear ester and combination with apple plant material. Different letters indicate statistically significant differences between the treatments (General Linear Model with binomial distribution and Tukey-Kramer pairwise comparisons, $P < 0.05$).

Earlier attempts to detect pear ester in apples had not been successful (Landolt & Guédot, 2008). Therefore, it is conceivable that plants may not constitutively produce this compound. Another source of semiochemicals, frequently overlooked, are the microbial communities associated with insect habitats (Davis *et al.*, 2013). Considering the strong mutualism between codling moth and apple yeasts (Witzgall *et al.*, 2012), we investigated the microbial communities associated with pear ester-emitting apples (Figure 11). Our results indicate a strong correlation between the emission of pear ester from apples and the presence of the yeast *Metschnikowia fructicola* and bacteria of the genus *Pantoea. Metschnikowia* yeasts have been shown to produce pear ester (Bengtsson *et al*., unpublished). However, we were unable to obtain pear ester from *M. fructicola* during controlled fermentation on minimal medium. We speculate that *M. fructicola* may need the co-habitation with other members of the microbial community (such as *Pantoea* spp.) to produce this kairomone. Similar results have been reported in the fruit fly: co-cultures of yeasts and bacteria produce a different and more attractive volatile profile than microorganisms grown individually (Fischer *et al.*, 2017). These results emphasize the importance of the interaction of microbial metabolites in chemical communication of insects (Chapter VI).

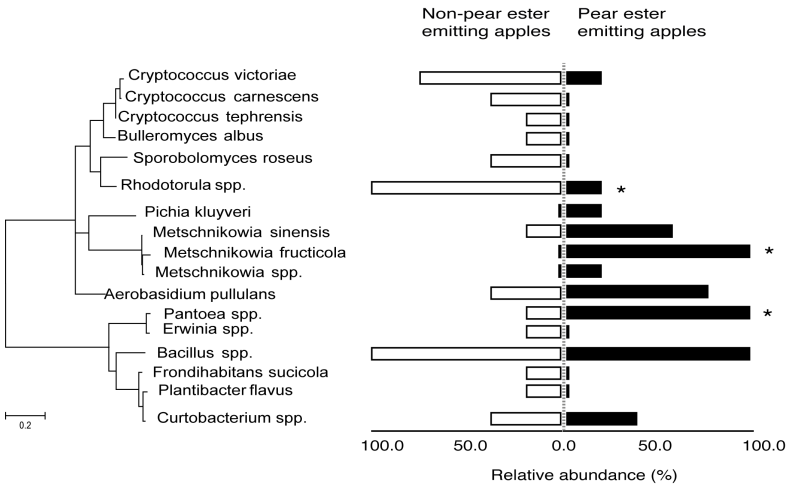

Figure 11. Microbial community composition of apples with and without pear ester emission. Asterisks indicate statistically significant correlation between the indicated microorganism and the presence or absence of pear ester emission (Fisher-exact Test, $p < 0.05$)

Regarding the second insect studied in this thesis, *Drosophila melanogaster,* we identified a novel long-range pheromone, (*Z*)-4-undecenal (Z4-11Al). This pheromone is produced by the spontaneous oxidation of the female-produced cuticular hydrocarbon, (*Z,Z*)-7,11-heptacosadiene. Recordings from native sensilla (ab9) and subsequent functional characterization of the receptors encoded by the gene OR69a demonstrated several important features of the perception of this pheromone. First, the gene OR69a presents two alternative spliced variants: OR69aA and OR69aB. Each of these variants has a different ligand specificity spectrum for different plant and yeast-related VOCs, and OR69aB is responsible for the detection of the pheromone Z4-11Al (Figure 12A). Behavioural tests further demonstrated species-specific bisexual attraction of a cosmopolitan *D. melanogaster* strain towards Z4-11Al, while the partially sexual-isolated strain Zimbabwe and the sister species, *D. simulans*, were not attracted (Figure 12B). Therefore, the gene OR69a encodes receptors detecting both food and social signals, mediating sexual isolation (Chapter VII).

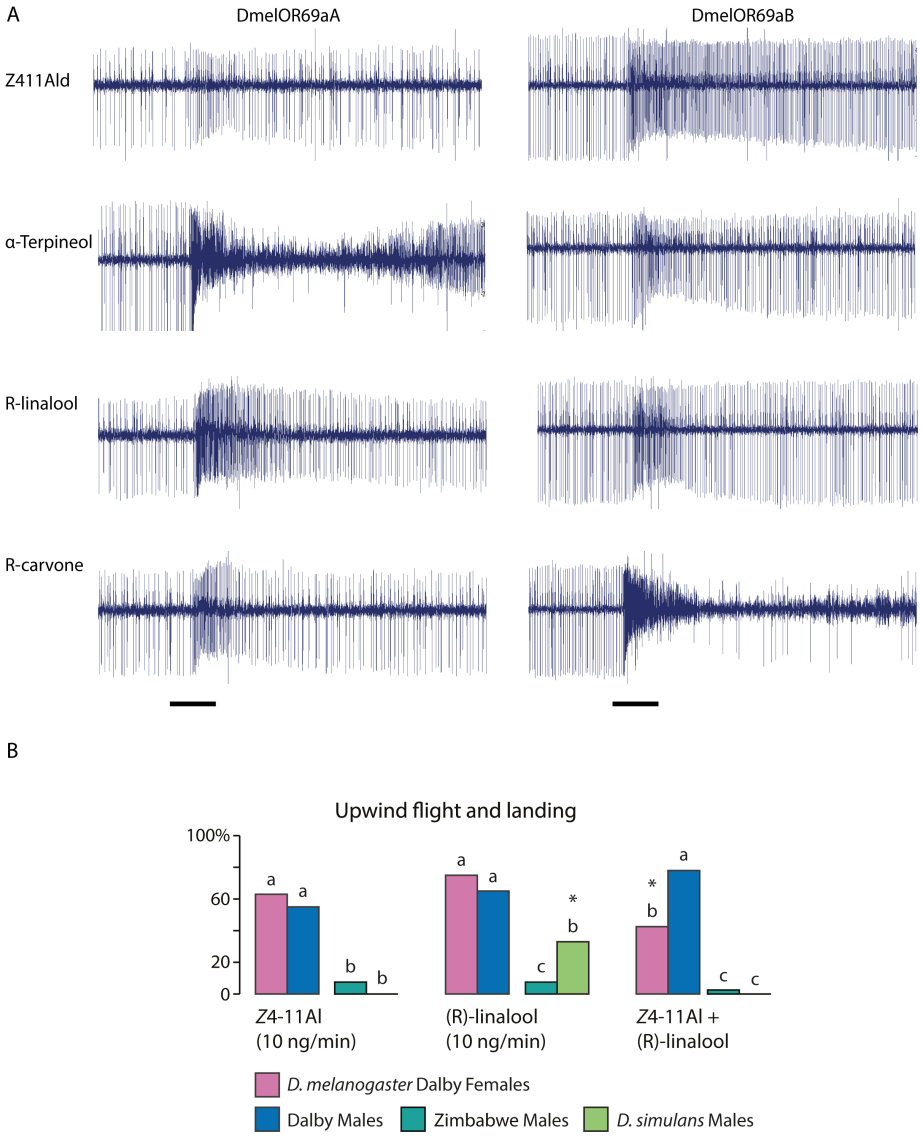

Figure 12. Response of flies towards the social and habitat cues. A) Response of neurons expressing alternatively spliced variants of DmelOR69a towards Z4-11Al and other ligands. B) Wind-tunnel assays of attraction of different strains and species of fruit flies. Letters indicate statistical differences between test insect strains and species, for each treatment. Asterisks indicate significant differences between treatments (n = 40, $P < 0.001$, binomial GLMs followed by post-hoc Wald pairwise comparison tests).

Taken together, both models represent examples of the elegant ways in which evolution has shaped mechanisms of detecting two types of signals, pheromones and kairomones. In the case of codling moth, previous electrophysiological recordings of the sensilla type *s. auricillicum* subtype rabbit eared-shoehorn indicated a dual response to pear ester and codlemone (Ansebo *et al.*, 2005). Therefore, it is possible that CpomOR3 and the codlemone receptor (putatively CpomOR1) may be colocalized in independent OSNs housed in the same sensillum. A new morphological study of codling moth antennae have shown the bisexual abundance of a type of trichoid sensilla in codling moth (Roh *et al.*, 2016). Although not characterized yet, these sensilla may house the co-localized neurons expressing receptors for these signals. Co-localization has been proposed as a mechanism of signal modulation (Andersson *et al.*, 2010), enhancing of ratio detection of odour mixtures (De Bruyne & Baker, 2008) and improvement of spatiotemporal resolution of chemical signals (Binyameen *et al.*, 2014; Baker *et al.*, 1998). It is conceivable that co-localization of neurons expressing receptors dedicated to food cues in the same sensilla as OSNs expressing PRs might serve as an extremely sensitive strategy to convey a strongly interconnected signal to the antennal lobe (Andersson *et al.*, 2010; Trona *et al.*, 2010a; Baker, 2009; Krieger *et al.*, 2009). In the case of *D. melanogaster*, simultaneous detection is achieved through co-expression of the two alternative spliced variants of OR69a in the membranes of the same OSN. Co-expression of different receptors in the same OSN has been shown in *Drosophila* and in *Anopheles* mosquitoes (Karner *et al.*, 2015; Ray *et al.*, 2007; Couto *et al.*, 2005; Goldman *et al.*, 2005; Dobritsa *et al.*, 2003). Goldman *et al.* (2005) postulated that co-expression of ORs, and hence simultaneous perception of different signals, may encode a different message in the antennal lobe, than when the signals are perceived separately. To the best of our knowledge, our study is the first report in which one of the co-expressed receptors is a PR, which reinforces the idea that coordinated perception of pheromones and habitat cues is of ecological and behavioural relevance.

4.3 Part 3: Prediction of Conserved ORs

Investigating the evolution of chemosensory receptors and their functions is an ultimate objective in Chemical Ecology. This research leads to a better understanding of the distribution and hosts shifts of insects. Additionally, it may facilitate the development of new control methods of species that conflict with human welfare (Raguso *et al.*, 2015).

Based on the insights gained researching codling moth ORs, we performed another chemosensory-targeted transcriptomic study comparing different tortricid species of agricultural importance. We predicted and quantified the expression of the three main gene families of chemosensory receptors expressed in male antennae of the green budworm moth, *Hedya nubiferana* H. (*dimidioalba* R.), the beech moth, *Cydia fagiglandana* Z., and the pea moth, *Cydia nigricana* F. Phylogenetic relationships, molecular evolutionary rate comparisons and transcript abundance estimation showed substantial similarities between these species and *C. pomonella*. Based on our experience with codling moth, we predict ligands of highly expressed orthologous ORs and PRs. For instance, the corresponding orthologs of CpomOR6a (the receptor of codlemone acetate) were highly expressed in both *Cydia* species, which correlates with the fact that codlemone acetate is their main pheromone component. Similarly, orthologous receptors of CpomOR3 (pear ester receptor) were found in *H. nubiferana* and in *C. fagiglandana,* which is consistent with field attraction of those species to pear ester (Jósvai *et al.*, 2016; Schmidt *et al.*, 2007). Furthermore, evidence of purifying selection (dn/ds < 1), supported our claims of conserved functions for the orthologs of CpomOR1, OR3 and OR6a (Figure 13). Since chemosensory adaptation is one of the factors mediating speciation, these results provide important insights on how semiochemicals may drive the host preference of these species (Chapter VIII).

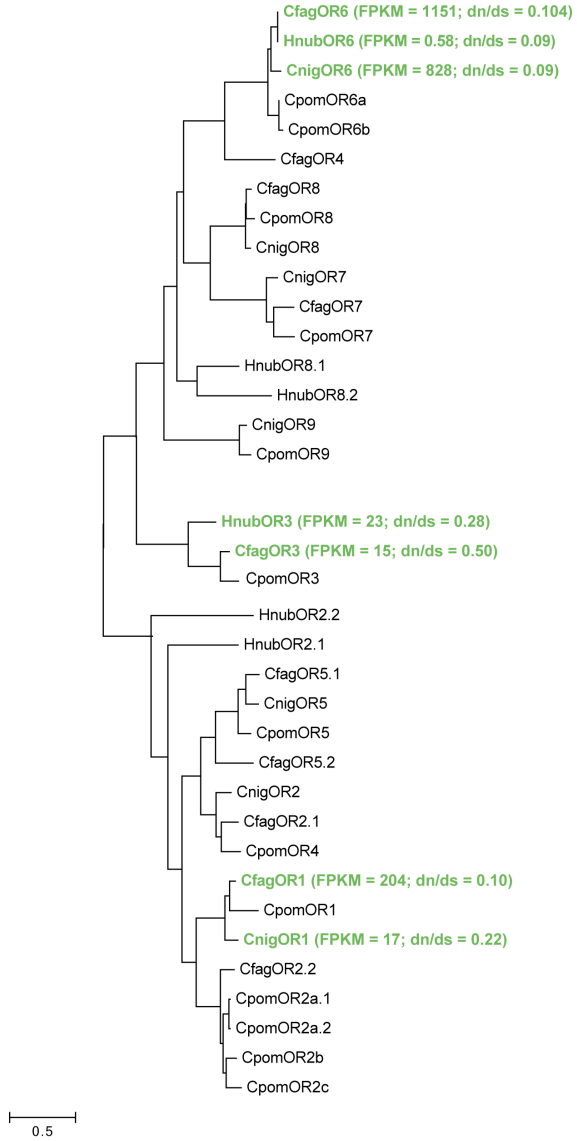

Figure 13. Phylogeny of PRs from four tortricid species. Selected orthologs are highlighted in green font. Abundance estimation (FPKM value) and molecular rate comparisons of selected orthologs with *Cydia pomonella* (dn/ds) are indicated between parentheses.

5 Concluding Remarks and Perspectives

Chemoreceptors, as detectors of chemical signals, reflect the chemical spaces in which insects live. Insects perceive sexual and environmental cues as an ensemble. Their combined perception may encode reproductive isolation and drive speciation.

In this thesis, I have shown the existence of a highly expressed, dedicated receptor for the detection of the kairomone pear ester, in codling moth, indicating a specific role as host cue (Papers I, II, III and VI). The putative conservation of this receptor in *Hedya nubiferana* and *Cydia fagiglandana,* (Paper VIII), may be an indicator of the emission of pear ester from other hosts. A correlation of the occurrence of pear ester with presence of microbes, *Metschnikowia fructicola* and *Pantoea* spp., indicates a microbial origin of this semiochemical. Functional characterization of the orthologous receptors of OR6 (Paper III) and OR19 (Paper IV) predicted in other tortricids (Paper VIII) may also provide better insights in the association between chemical-structure activity and the evolutionary forces shaping the divergence of olfactory receptors. To this purpose, we showed that the heterologous expression using the OR22a-Gal4 mutants to deorphanize general odourants and the OR67d-Gal4 lines to deorphanize pheromone receptors, are viable experimental approaches (Paper V). Further studies, however, should include the expression of SNMPs and OBPs for recalcitrant receptors such as CpomOR1.

In the case of *Drosophila melanogaster*, we characterized the non-overlapping and unique responses of two alternatively spliced variants of the receptor OR69a, with the peculiarity that one of these variants responds to a newly reported long-range aggregation pheromone, Z4-11Al (Paper VII). Further study of the functional evolution of this receptor may provide insights into the evolution of drosophilid flies and may lead to the development of control techniques against the hazards of pest species such as *Drosophila suzukii*.

The findings in this thesis accentuate two lines of future research. First, the strategies through which insects integrate the smells of sociality (kairomones and pheromones), from the moment of perception until their integration at the antennal lobe, need further characterization. Additional experimental work is required to clarify these interactions, and new tools such as the CRISPR/Cas9 gene editing system hold promise for investigating model and non-model organisms alike. Second, another aspect of research that needs to be taken into deeper consideration is the role of microbes in chemical communication. Both the fruit fly and codling moth are strongly associated with microorganisms, but we still do not know how microbial communities are structured in habitats and how they contribute to host finding in addition to plant VOCs. Microbial profiling through next generation sequencing (NGS) methods, such as the Illumina Sequencing, may help in correlating insect semiochemicals and specific microorganisms.

We know now that kairomones and pheromones are perceived together. Perhaps, in a near future, we will demonstrate that kairomones are the result of complex interactions between plant and microbial components.

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