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The Evolution of Sex Pheromone Reception in Sibling Species of the New Zealand Endemic Leafroller Moth Genera *Ctenopseustis* and *Planotortrix*

Bernd Steinwender

A thesis submitted in the fulfilment of the requirements of Doctor of Philosophy in Biological Science
Abstract

How new mate recognition systems evolve when changes are required in both the male and female components remains a conundrum. Here we investigate the ultrastructure of male and female antennae and the molecular basis of pheromone reception in two pairs of closely-related species of tortricid (leafroller) moth, *Ctenopseustis obliquana* and *C. herana* as well as *Planotortrix octo* and *P. excessana*.

We identified six types of sensilla on the ventral surface of their antennae using scanning electron microscopy: S. trichodea (type I, type II, type III), s. basiconica, s. auricillia, s. chaetica, s. coeloconica and s. styloconica. No major differences in gross antennal morphology or number and type of sensilla were found among the four species. Sexual dimorphism was observed, with sensilla trichodea (type I) only found in males. This finding is consistent with a role for these sensilla in detecting the female sex pheromone reported in other moth.

Using a transcriptome sequencing approach from adult male and female antennae, we identified 47 olfactory receptors (ORs) from each *C. obliquana*, *C. herana* and *P. excessana* and 48 ORs from *P. octo* and assessed their expression levels in male and female antennae using RNA seq-counting and quantitative RT-PCR (qPCR). By using qPCR we identified three male biased and one female biased OR in *C. obliquana*, four male biased and one female biased OR in *C. herana*, two male biased and one female biased OR in *P. octo* and two male biased and one female biased OR in *P. excessana*. Two ORs were male biased in all four species, OR7 and OR30. Candidate pheromone receptors were tested for their ability to respond to sex pheromone components in a HEK293 cell calcium assay. CoblOR7 and CherOR7 responded to (Z)-8-tetradecenyl acetate (Z8-14:OAc), a pheromone component produced by *C. obliquana* females. CherOR7 has a reduced sensitivity to Z8-14:OAc and unlike CoblOR7, also responds (Z)-7-tetradecenyl acetate (Z7-14:OAc), which is not a pheromone component in either of the two species, indicating that CherOR7 may be under relaxed constraint compared with CoblOR7. Significantly higher sequence differences were found in the third and the sixth transmembrane domain region of CoblOR7. PAML analysis conducted on orthologous ORs of all four species indicate positive selection acting on the male biased OR7 and another OR, OR64. The fact that OR7 is likely under positive selection, that it is male biased in its expression and that its orthologue in *C. obliquana*, CoblOR7, responded to sex pheromone components is suggestive of this receptor being important in sex pheromone reception in *Planotortrix* species also.
Acknowledgements

The journey to my PhD was a long and sometimes difficult but certainly not a lonely one. Many people supported, influenced and encouraged me through these years and kept me going.

First of all I want to thank my supervisor Richard Newcomb. His patience and friendly advice helped me tremendously. He surprised me over and over again with his knowledge and the amount of people he knew. I couldn’t wish for a better mentor to guide me. I thank you so much Richard for giving me the opportunity to come to New Zealand from the other side of the world and fulfil my life goal of becoming scientist.

I was lucky enough to work with an amazingly crazy bunch of people who are weird and awesome in their ways. Thank you for the laughter, the inspiring, intellectual, obscure and also silly conversations we had and the drinks we shared Colm, Jeremy, Cyril, Emma, Andrew, Jerome, Jacob, Melissa, Ed, Malcolm and John. Especially I want to thank Selene for her awesome sense of humour and her craziness and Leah for the deep and meaningful conversations combined with highly inappropriate chats. Also, thank you Leah, because of you I felt like my swearing wasn’t too bad.

A huge thank you goes to Anne Barrington. She didn’t only supply me with moths, she also was my shoulder when I had a bad day. Even though she tried to scare me away but it never worked. Sorry that I didn’t have much time in the last months of my PhD but in thoughts I was always annoying you. You are an awesome chick!

Coming to New Zealand all by myself, meant also that I didn’t know anyone and that I needed to find a place to live. I was lucky enough to find not only a place to stay but also people I call my “New Zealand Family”. Thank you Taz and Karen Darragh for everything you’ve done for me. You not only provided me with a room to stay but made me part of your family. You are the kindest and nicest people I have ever met. You were there whenever I needed someone and you surely helped me through some tough times. I love you both and beautiful Sofia. I couldn’t have done it without you.

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During the last year of my PhD I started a new hobby called CrossFit. With the exercise and training comes an amazing community and I have been part of this very community since I started CrossFit. I
want to thank the members and coaches of CrossFit Newmarket for the training, the sweat and the fun. The life of a PhD student can be lonely but you as part of a community like this. All of you are awesome and there are too many people to name but you know who you are (D55)!

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<td>µl</td>
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<td>14:OAc</td>
<td>Tetradecenyl acetate</td>
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1.
General introduction

The ability to smell and to distinguish different kinds of odours is crucial for all animals. Insects use their sense of smell to orientate within their environment and to communicate with other individuals which is important for survival. Communication involves the transfer of information from the sender to the receiver and the receiver decides how to respond to the signal (Bradbury and Vehrencamp 1998). In general, signals are beneficial for the sender, transferred voluntarily, and their information is also of benefit for the receiver. Information can be transferred via sound, light or chemical signals (Bradbury and Vehrencamp 1998). It is the transfer of information (i.e. communication) via chemical signals between tortricid moths that is the subject of discussion in this thesis.

Chemical signals are called pheromones and are by definition substances that are released to the outside (Karlson and Butenandt 1959). Pheromones are broadly used by insects especially when it comes to communication between individuals of the opposite sex (Karlson and Butenandt 1959).

In moths (Lepidoptera) sex pheromones are used by females to attract males over long distances (Linn and Roelofs 1995). Males locate females through upwind flight along a concentration gradient of the sex pheromone (Kaissling 1997). Species specificity in regards to pheromone production and reception is important and forms a robust mate recognition system that limits incompatible mating events (Linn and Roelofs 1995) as mating mistakes can be fatal for both male and female (Stadelbacher et al. 1983). It seems difficult for mating systems that rely on chemical cues to evolve rapidly because purifying selection should prevent drastic changes in either pheromone production or reception. A hypothesis that attempts to solve this dilemma is the “asymmetric tracking hypothesis”. This model suggests that mate-signalling systems are only rarely under strong mutual stabilising selection (Phelan 1992). In systems where females are the limiting sex, greater variation may be tolerated in the male’s pheromone reception system to provide a scenario where rare males may exist that are able to sense a novel pheromone blend (Phelan 1992). Such males can be found in *Ostrinia nubilalis* where rare males are attracted to the pheromone blend of the closely related species *Ostrinia furnacalis* (Domingue et al. 2007).

In tortricid moths, and insects in general, odorants are perceived mainly with the antennae, which are covered with hair-like sensilla of different types. The antenna of a tortricid moth typically consists of three components: scape, pedicel and flagellum (Albert and Seabrook 1973; Wall 1978; Gomez and Carrasco 2008). The scape and pedicel consist only of a single segment each but the flagellum consists of several subsegments. The number of subsegments varies among tortricid moths ranging from 34 to 39 in *Talponia batesi* (Gomez and Carrasco 2008) to more than 50 subsegments in *Cydia nigricana* (Wall 1978). The antennae are covered with a variety of sensilla. Typically in tortricids six
different types of sensilla are present on the antennae: Sensilla trichodea, sensilla basiconica, sensilla auricillia, sensilla chaetica, sensilla coeloconica, sensilla styloconica (Razowski and Wojtusiak 2004). The various types of sensilla fulfil various sensory tasks such as detection of chemicals (chemoreceptor), mechanical stimuli (mechanoreceptor), moisture (hygroreceptor) and changes in temperature (thermalreceptor) (Albert and Seabrook 1973; Hallberg 1981; van der Pers et al. 1980; Pophof 1997). Sensilla trichodea for example are involved in sex pheromone detection in moths (den Otter et al. 1978; Rumbo 1981, 1983; Hansson et al. 1989; Krieger et al. 2005) and s. coeloconica has been shown to respond best to acids and aldehydes in *Bombyx mori* (Pophof 1997). While the function of some sensilla such as s. trichodea or s. coeloconica has been unravelled in some species, the function of many sensilla is still unclear.

The sensillum as the smallest functional sensory unit in insect olfaction (Keil 1999) contains olfactory receptor neurons (ORNs) that generally express a single olfactory receptor (OR) gene (Vosshall et al. 2000). Insect OR proteins are located within the ORN dendritic membrane (Figure 1) and contain seven transmembrane regions with their N-terminus located in the cytoplasm and an extracellular C-terminus (Figure 2) (Smart et al. 2008; Benton et al. 2006; Lundin et al. 2007).

![Figure 1](image)

**Figure 1.** A. The antennae of a male *Planotortrix octo*. B. Scanning electron micrograph of an antennal sub-segment displaying long sensilla trichodea that contain pheromone receptors. C. Schematic diagram of a sensillum showing odorants or pheromones entering the sensillum through pores. D. Close up of the dendrite expressing receptors that bind odorants or pheromones.
There is still controversy about the signal transduction mechanisms in insect olfaction. In total three different mechanisms have been suggested. The first hypothesis suggests that signal transduction involves a heterotrimeric G-protein-mediated second messenger pathway (Figure 3A) (Krieger and Breer 1999; Jacquin-Joly and Merlin 2004). This model however has been largely rejected and there is more evidence that ORs build heteromeric complexes with the odorant receptor co-receptor (Orco) (Benton et al. 2006) that is always expressed in the ORN (Voshall et al. 2000). It has been shown that Orco forms a heteromeric complex with the OR (Benton et al. 2006). A co-expression of heteromeric complexes of insect ORs and Orco showed the properties of a cation non-selective ion channel directly gated by odour or pheromone ligands (Figure 3B) (Sato et al. 2008; Touhara 2009). The third model suggests rapid transient and slow prolonged currents. The fast currents are consequences of direct Orco/OR interaction. The slow currents on the other hand happen through a G protein activation of Orco which is slower but more sensitive (Figure 3C) (Wicher et al. 2008; Stengl and Funk 2013). Further investigation of the possible mechanisms of signal transduction of odorants and sex-pheromones needs to be done to verify the right pathway.

ORs specialised in binding sex-pheromones are called pheromone receptors (PRs) and are located in s. trichodea (Rumbo 1981, 1983; Krieger et al. 2005). This family of receptor genes is generally more highly expressed in males than in females (Grosse-Wilde et al. 2010; Krieger et al. 2005; Krieger et al. 2004) which is also congruent with sex biased expression of s. trichodea (Grosse-Wilde et al. 2010; Jordan et al. 2008; Koh et al. 1995; Gomez and Carrasco 2008). In the past 15 years advancement in sequencing technology and new molecular biology tools made it possible to identify genes encoding ORs and PRs. One of the best studied moths in respect to olfaction is the silkmoth, *Bombyx mori*. Forty nine ORs were identified in this moth (Anderson et al. 2009) and BmOR-1 was functionally characterised as a PR responding to the pheromone component Bombykol (Sakurai et al. 2004). Apart from the domesticated *B. mori* as a model organism, ORs of many other moths have been investigated.
as part of research focused on finding novel ways to control the population size of pest species using olfactory-based techniques. Among the species investigated were *Heliothis virescens* (Krieger et al. 2002), *Manduca sexta* (Grosse-Wilde et al. 2011), *Cydia pomponella* (Bengtsson et al. 2012) and *Epiphyas postvittana* (Corcoran 2014). Even though PRs have been identified and also characterised in different species such as *B. mori* (Sakurai et al. 2004), *H. virescens* (Wang et al. 2011), *O. nubilalis* (Wanner et al. 2010) and *E. postvittana* (Corcoran 2014) we still don’t know much about how new mating systems arise.

**Figure 3.** Different possible mechanisms for signal transduction of odorants and sex pheromones A. This model proposes insect olfaction through a G-protein-mediated pathway. B. In this model the odorant receptor (OR) and the odorant receptor co-receptor Orco form a heteromeric odorant-gated non-selective cation channel. C. Here two pathways are described involving Orco as a cation channel. The first pathway is described as a fast but transient cation influx through the direct activation of Orco by an OR that binds a ligand. The second describes a metabotropic pathway and is coupled to a G-protein that induces a slow but prolonged cation current. Figure taken from Sakurai et al. 2014.

In a comparison of ORs from eight different lepidoptera species (*Danaus plexippus*, *Heliconius melpomene*, *Spodoptera littoralis*, *Manduca sexta*, *Bombyx mori*, *Heliothis virescens*, *Cydia pomonella*, *Plutella xylostella*) it has been shown that the molecular evolution of ORs and the expansion of gene families might be associated with the adaptation to host plants (Figure 4) (Engsontia et al. 2014). Sex pheromone receptor proteins showed monophyletic relationships within the clade as well as conserved amino acid sequences in their receptor proteins (Figure 5). This and male biased expression suggests that sex pheromone communication using these kind of receptors
seems to be an ancient phenomenon (Engsontia et al. 2014). It is suggested that during the evolution of lepidoptera chromosomal translocation happened but receptors kept the function. Also gene duplications in sex specific lineage have been detected. Engsontia et al. hypothesise that positive selection sites on the sex pheromone receptors could correlate with sex pheromone response specificity.

Figure 4. Phylogenetic tree of eight lepidopteran species: Danaus plexippus, Heliconius melpomene, Spodoptera littoralis, Manduca sexta, Bombyx mori, Heliothis virescens, Cydia pomonella, Plutella xylostella. Tree constructed from amino acid sequences using Bayesian analysis. Tree is rooted to Orco. Figure taken from Engsontia et al. 2014, details can be found within.
It has also been found that ORs show considerable differences in the rate of evolution. A comparison of three orthologous ORs in New Zealand native leafroller moth species *C. obliquana*, *C. herana*, *P. octo*, *P. excessana* and *P. notophaea* (Carraher et al. 2012) revealed that Orco is highly conserved among all investigated species. This can be explained by the fact that Orco is essential for olfaction in insects. The second investigated receptor OR3 is also highly conserved and it is speculated that is has an important role in binding citral. Unlike Orco and OR3, OR1, the third receptor investigated in this study, seemed to evolve much faster. OR1 can be found in the so called sex pheromone receptor clade and it is still not entirely clear why there is such a high rate of evolution within that clade. The finding that ORs in general seem to evolve in different rates have also been described in Drosophila (Guo and Kim 2007; McBride et al. 2007; de Bruyne et al. 2010).

**Figure 5.** Predicted topology of a sex pheromone receptor protein with its seven transmembrane regions. The circle and squares represent amino acid residues. Gray circles represent conserved amino acid sites shared among members of the OR clades. Black squares represent amino acids that evolved under positive selection (P > 50 %); * and ** indicate P > 90 and 95 %, respectively. Figure taken from Engsontia et al. 2014
A way of studying the evolution of sex-pheromone production and perception is to examine and compare relevant genes from closely related species or strains within the same species that use different sex pheromones as found in the European corn borer (ECB) *O. nubilalis* (Anglade and Stockel 1984).

New Zealand endemic leafroller moths consist of a number of closely related species that also offer the potential to carry out such a study and were therefore the subject of this thesis. In the following paragraphs I review in detail what we know about species within the genus *Ctenopseustis* and *Planotortrix*.

**The New Zealand endemic leafroller moths**

The species within *Ctenopseustis* and *Planotortrix* differ in their distribution across New Zealand. While some species such as *C. obliquana*, *P. excessana* and *P. octo* are widespread throughout the country, others are more restricted. *Ctenopseustis herana*, *C. filicis* and *P. puffini* are restricted to the South Island, *C. fraterna* occurs only on the North Island and *P. octoides* is found only on the Chatham Islands (Dugdale 1990). The species also differ in host specificity. *Ctenopseustis fraternal*, *C. ficilis*, *P. avicenniae* and *P. puffini* are specialised on one or only a few plants while *C. servana*, *C. obliquana*, *C. herana*, *P. excessana*, *P. octo* and *P. octoides* are polyphagous (Dugdale 1990). The polyphagous species *C. obliquana* (Figure 6 A and B), *C. herana* (Figure 7 A and B), *P. excessana* (Figure 8 A and B) and *P. octo* (Figure 9 A and B) are pest species in the complex damaging crops such as pipfruit, summerfruit and kiwifruit (Wearing et al. 1991).

![Figure 6. Images of the New Zealand endemic leafroller moth species *Ctenopseustis obliquana* from dorsal, ventral and lateral view A. male and B. female.](image)
Adult moths range in size from 10 to 15 mm, with *Ctenopeustis* measuring slightly smaller than *Planotortrix*, and the males in general smaller than females. Wing patterns are highly variable, both within and between species. Up to four generations per year are possible in all *Ctenopeustis* and *Planotortrix* species (Dugdale 1990). In both genera larvae overwinter as non-diapauing larvae with a possible additional generation during winter on the warmer North Island in June or July (Wearing et al. 1991). Females lay batches of up to 600 eggs and larvae emerge after 8-9 days. Larvae have five to six instars with a mean larval period of 32 days for males to 36 days for females (Clare and Singh 1988).

In the late 1970s studies were executed to identify the pheromones of the green-headed leafroller (GHL), *P. excessana*, and the brown-headed leafroller (BHL), *C. obliquana* resulting in the identification of unsaturated sex pheromone components (Z)-8- tetradecenyl acetate (Z8-14:OAc) for the GHL (Galbreath et al. 1985), and an 80: 20 mixture of Z8-14:OAc and (Z)-5- tetradecenyl acetate (Z5-14:OAc) for the BHL (Young et al. 1985). Interestingly field cage experiments with progeny of two distinct populations of GHL resulted in females only attracting males from the respective population. Preliminary analysis of pheromone glands unravelled different tetradecenyl acetates, namely Z5-14:OAc and (Z)-7-tetradecenyl acetate (Z7-14:OAc) were produced in the pheromone glands of females of these two populations which has been confirmed in field tests using synthetic compounds (Galbreath et al. 1985). In the mid 1980s a large study was conducted to determine whether there are additional GHL and BHL pheromone types. For this project females from all over New Zealand were analysed for pheromone content and individuals were identified and characterised by specialists. The reassessed taxonomy of both genera revealed five species in *Ctenopeustis* and seven in *Planotortrix* (Dugdale 1990) (for pheromone blends see Table 1).

![Figure 7. Images of the New Zealand endemic leafroller moth species *Ctenopeustis herana* from dorsal and ventral view A. male and B. female.](image)
Table 1. Pheromone blends of the species within the genera *Ctenopseustis* and *Planotortrix* consist generally of unsaturated tetradecenyl acetates (14:OAc) but unsaturated hexadecenyl acetates (16:OAc) can also be found in some species.

Molecular markers with morphological and pheromone characters were used to explore the speciation events within the group. Phylogenetic studies concentrating on the mitochondrial cytochrome oxidase gene confirmed the monophyly of the genera *Ctenopseustis* and *Planotortrix* (Newcomb and Gleeson 1998; Gleeson et al. 2000; Langhoff et al. 2009). *Ctenopseustis servana* is positioned basally compared to the other species but how the other four species in *Ctenopseustis* relate to each other still needs to be resolved. In *Planotortrix*, two separated groups could be identified, one that consisted of *P. flammea*, *P. notophaea* and *P. puffini* and another group fitting the remaining four species, *P. octo*, *P. octoides*, *P. excessana* and *P. avicenniae*. The species within these two groups were little resolved (Langhoff et al. 2009).

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex pheromone components</th>
<th>Ratio</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Planotortrix excessana</em></td>
<td>Z5-14:OAc, Z7-14:OAc</td>
<td>60:40</td>
<td>Foster et al., 1989, Galbreath et al. 1985</td>
</tr>
<tr>
<td><em>Planotortrix octo</em></td>
<td>Z8-14:OAc, Z10-14:OAc</td>
<td>98:2</td>
<td>Foster et al., 1986, Galbreath et al. 1985</td>
</tr>
<tr>
<td><em>Planotortrix avicennia</em></td>
<td>Z5-14:OAc</td>
<td>100</td>
<td>Foster &amp; Roelofs, 1987</td>
</tr>
<tr>
<td><em>Planotortrix octoides</em></td>
<td>Z8-14:OAc</td>
<td>100</td>
<td>Dugdale, 1990</td>
</tr>
<tr>
<td><em>Planotortrix puffini</em></td>
<td>Z5-14:OAc, Z7-14:OAc, Z9-14:OAc</td>
<td>3:97:2</td>
<td>Foster &amp; Dugdale, 1988</td>
</tr>
<tr>
<td><em>Planotortrix flammea</em></td>
<td>Z5-14:OAc, Z7-14:OAc</td>
<td>52:48 to 61:39</td>
<td>Foster et al., 1990</td>
</tr>
<tr>
<td><em>Planotortrix notophaea</em></td>
<td>Z7-14:OAc, 18-Ald, 14-OAc, Z7-14:OH, 16-Ald</td>
<td>100:11:5:3:3</td>
<td>Foster et al., 1986; A. El Sayed, unpublished data</td>
</tr>
<tr>
<td><em>Ctenopseustis obliquana</em></td>
<td>Z8-14:OAc Z5-14:OAc</td>
<td>80:20 to 90:10</td>
<td>Foster et al., 1986; Young et al., 1985</td>
</tr>
<tr>
<td><em>Ctenopseustis herana</em></td>
<td>Z5-14:OAc</td>
<td>100</td>
<td>Foster &amp; Roelofs, 1987</td>
</tr>
<tr>
<td><em>Ctenopseustis fraterna</em></td>
<td>16 carbon acetate diene</td>
<td>?</td>
<td>B. Morris, unpublished preliminary data</td>
</tr>
<tr>
<td><em>Ctenopseustis filicis</em></td>
<td>Z10-16:OAc</td>
<td>100</td>
<td>Foster &amp; Dugdale, 1988</td>
</tr>
<tr>
<td><em>Ctenopseustis servana</em></td>
<td>Z5-14:OAc, Z7-14:OAc</td>
<td>32:68 to 35:65</td>
<td>Foster &amp; Dugdale, 1988; Foster et al., 1990</td>
</tr>
</tbody>
</table>

Figure 8. Images of the New Zealand endemic leafroller moth species *Planotortrix octo* from dorsal and ventral view A. male and B. female.
Since the discovery of the different sex pheromone blends within the New Zealand endemic leafroller complex research has focused on understanding the biosynthesis of these compounds and biochemistry involved in producing the different pheromone blends in the pest species C. obliquana, C. herana, P. octo and P. excessana (pheromone blends and molecules of these species can be found in Table 2). Analysis with deuterium-labelled saturated fatty acids in the pheromone gland of P. octo showed that a ∆10-desaturase was the key enzyme in the biosynthesis of Z8-14:OAc (Foster and Roelofs 1988). In P. excessana the key enzymes for the production of the two pheromone components Z5-14:OAc and Z7-14:OAc are two ∆9-desaturase enzymes (Foster et al. 1989) encoded by two different genes (Albre et al. 2012). Although closely related Z5-14:OAc in C. herana is produced through ∆5-desaturation (Foster and Roelofs 1996). Molecular studies on pheromone gland desaturases in C. herana and C. obliquana have identified genes coding for ∆10- and ∆9-desaturases (Albre et al. 2012), and most recently a ∆5-desaturase (Hagstrom 2013). However, the specific enzymes involved in the biosynthetic pathway of Z5-14:OAc and Z8-14:OAc in C. obliquana have not been characterised (Figure 10 shows the basic biosynthetic pathway for the production of pheromone components used in C. herana, C. obliquana, P. octo and P. excessana).

Table 2. Pheromone blends and chemical structure of compounds used in the species Planotortrix excessana, P. octo, Ctenopseustis obliquana and C. herana

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex-pheromone components</th>
<th>Ratio</th>
<th>Chemical structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planotortrix excessana</td>
<td>Z5-14:OAc, Z7-14:OAc</td>
<td>60:40</td>
<td></td>
</tr>
<tr>
<td>Planotortrix octo</td>
<td>Z8-14:OAc, Z10-14:OAc</td>
<td>90:2</td>
<td></td>
</tr>
<tr>
<td>Ctenopseustis obliquana</td>
<td>Z5-14:OAc, Z8-14:OAc</td>
<td>90:10</td>
<td></td>
</tr>
<tr>
<td>Ctenopseustis herana</td>
<td>Z5-14:OAc</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Figure 9. Images of the New Zealand endemic leafroller moth species Planotortrix excessana from dorsal and ventral view A. male and B. female.
**Figure 10.** Simplified outlining of the biosynthetic pathways of the sex pheromone components of *Ctenopseustis obliquana*, *C. herana*, *Planotortrix octo* and *P. excessana*. Desat1 is the desaturase gene encoding a Δ9-desaturase with a preference for 16>18 carbon fatty acids. Desat5 and desat6 correspond to desaturase genes encoding a Δ10-desaturase and a Δ9-desaturase with a preference for 18>16 carbon fatty acids, respectively. Desat7 has recently been identified to be desat7 encoding a Δ5-desaturase. ‘-2C’ describes chain shortening by β-oxidation. Figure taken from Albre et al. 2012.

Since it is possible to hybridise sibling species in the laboratory, in *C. obliquana* and *C. herana* the genetic basis of sex pheromone production has been investigated (Foster et al. 1997). Females of *C. obliquana* produce a blend of Z8-14:OAc and Z5-14:OAc in a ratio of 80:20, whereas *C. herana* females produce only Z5-14:OAc. While F₁ females produced a pheromone blend that was similar to that of *C. obliquana*, females from F₁ backcrosses and F₂ crosses produced a complex array of blends. The F₂ offspring produced pheromone blends that would suggest autosomal inheritance, the paternal backcrosses generated phenotypes that best fitted a sex-linked model, and the maternal backcrosses did not fit any of the two models. Therefore multiple genetic factors are likely to be involved in female sex pheromone production in these species.

A study on species within the genus *Planotortrix*, *P. octo* and *P. excessana*, has been conducted more recently (Albre et al. 2013). Females of *P. octo* produce a blend consisting of Z8-14:OAc, whereas *P. excessana* females produce a blend of two different compounds, Z5-14:OAc and Z7-14:OAc. Here the pheromone blend of F₁ hybrids, all backcrosses and F₂ crosses resembled the *P. excessana* blend and hardly any Z8-14:OAc could be detected. The amount of Z8-14:OAc in the pheromone gland corresponded to the level of expression of the Δ10-desaturase, desat5. The segregation patterns...
observed in all crosses best fit a genetic model involving one major and other minor genes. The major gene is thought to be a dominant trans-acting repressor of desat5 expression in the female pheromone gland.

In both genera there has been a change in the use of Z8-14:OAc as a component of the sex pheromone blend. In C. obliquana Z8-14:OAc is the main component but not present in C. herana. In P. octo this compound is also the sole component but it is not found in P. excessana. In the species that use Z8-14:OAc as a pheromone compound, C. obliquana and P. excessana, the desat5 gene that encodes the Δ10-desaturase is highly expressed in the pheromone gland of females. The expression of orthologues of in C. herana and P. excessana however, is barely detectable even though present within their respective genomes (Albre et al. 2012). The production of specific pheromone blends used by the sibling species seems to be modified by a simple switching on and off of gene expression. The expression of desat5 in Planotortrix is controlled by a dominant trans-acting repressor present in P. excessana but absent in P. octo. A 7-bp long region, which is argued to be the promoter of desat5 in P. excessana, is associated with higher expression of the gene in the pheromone gland (Albre et al. 2013).

It has been suggested that in the genus Ctenopseustis there has been a gain in the use of Z8-14:OAc in the evolution of the genus after the split from C. servana. This gain in the expression of the Δ10-desaturase in the pheromone gland has become widespread and is used by C. obliquana but C. herana, which only produces Z5-14:OAc, appears to have lost Δ10-desaturase expression in its pheromone gland (Albre et al. 2012). In Planotortrix the evolution of sex pheromone components containing a Δ10- and Δ8-unsaturation are likely derived from a gain of expression of the Δ10-desaturase in the pheromone gland. Components such as Z5-14:OAc and Z7-14:OAc are widespread in the genus which indicates these are ancestral pheromone compounds. Z8-14:OAc is used by just two species, P. octo and P. octoides, what indicates a gain-of-function event probably through the release of repression by the P. excessana repressor of desat5 and therefore the gain of an activator binding site in the desat5 promoter region (Albre et al. 2013).

There has also been a discussion about the evolution of the Δ5- and Δ9-desaturases. The Δ9-desaturase that produces Z5-, and Z7-14:OAc is widespread in Planotortrix however in Ctenopseustis the desat7 gene which encodes for a Δ5-desaturase appears to be responsible for producing Z5-14:OAc in C. herana and maybe C. obliquana (Newcomb et al. 2014 in press).

The genetic basis of sex pheromone perception and its behavioural response in male C. obliquana and C. herana has also been investigated (Foster et al. 1997; Hansson et al. 1989). The electrophysiological study revealed, that the sensilla trichodea of C. obliquana males contained a large spike amplitude cell that responded strongly to the main pheromone component, Z8-14:OAc, and also a small spike amplitude cell that responded weakly to the minor component, Z5-14:OAc. In C. herana on the other hand, the large spike amplitude cell responded strongly to Z5-14:OAc, as the
main pheromone component and the small spike amplitude cell responded weakly to Z8-14:OAc. The differences in the electrophysiological response indicate a swapping of the sensitivity of the two sensory neurons in these species.

Crossing experiments between the two species produced F1 males responding similarly to *C. herana*. Apart from sensilla responding to Z5-14:OAc and Z8-14:OAc, some sensilla also responded to neither compound and were classified as “atypical” (Hansson et al. 1989). In general the electrophysiological response of crosses and backcrosses suggested a model of Z-linked inheritance with the *C. herana* type being dominant. The high variability in the response patterns of the hybrids and the presence of atypical receptor neurons, suggested that it is likely that more genes are involved.

Hybrids between *C. obliquana* and *C. herana* have also been tested in wind tunnel experiments (Foster et al. 1997). Different behaviours from wing-fanning to flying towards the pheromone source were scored and recorded from individual males. Males were tested with blends of Z8-14:OAc and Z5-14:OAc at ratios of 0: 100, 30:70, 70: 30 and 90: 10. Most *C. obliquana* males responded to the blends with the highest Z8-14:OAc ratio but didn’t respond to a pheromone blend consisting of Z5-14:OAc only. *Ctenopseustis herana* males responded highly to blends that consisted of Z5-14:OAc only or were low in Z8-14:OAc. F1 males responded more broadly than either of the respective parents with a higher proportion of males responding to blends containing more Z5-14:OAc. Parental backcrosses and F2s also showed rather broad response patterns but overall the response was similar to the electrophysiological experiments, suggesting Z-linked inheritance of a major gene, with *C. herana* as the dominant type, but also with other genes involved resulting in the observed variability of the responses.

So far only three ORs have been identified in *C. obliquana, C. herana, P. octo* and *P. excessana* using orthologues from *E. postvittana*. These receptors include the highly conserved OR co-receptor, Orco, a receptor that is related to pheromone receptors in other moths OR1 and a third OR3. A sequence comparison of these genes revealed that OR1 is evolving rapidly compared to the other two receptors (Carraher et al. 2012).
Research Aims

The goal of this PhD research is the identification and characterisation of PRs in the New Zealand endemic leafroller sibling species *C. obliquana*, *C. herana*, *P. octo* and *P. excessana* to gain more understanding of how sex pheromone perception in male moths has evolved. The sibling species are morphologically very similar, occur in sympatry, and are highly polyphagous. Although these species are not very different in many aspects they are reproductively isolated solely through their sex pheromones. As reviewed in the introduction a lot of work has been done on sex pheromone production and we are now beginning to understand how new pheromone blends are created in these species. However, not much is known about the male side of the equation. This makes the New Zealand native leafroller a good organism to study the evolution of sex pheromone perception.

The research carried out for this thesis can be divided into three sub-sections: the analysis of the ultrastructure of male and female antennae of all four species of New Zealand endemic leafroller moths, the identification of ORs, and the functional characterisation of candidate PRs, and testing identified ORs for possible positive selection.

At the beginning of this PhD project it was evident that there was no ultrastructure data available for the species within the New Zealand endemic leafroller complex. I hypothesised that differences in the ultrastructure could correspond to differences in the preference for distinct sex pheromone components. Additionally, I wanted to investigate whether the antennae of *Ctenopseustis* and *Planotortrix* species exhibit sexual dimorphism. At the Plant and Food Research Mt Albert there is a scanning electron microscope available which was used to examine the antennae of the species *C. obliquana*, *C. herana*, *P. octo* and *P. excessana*.

In female moths differences in the production of sex pheromones is associated with the expression of desaturase genes in their pheromone gland (see above). In males a similar genetic mechanism could be responsible for the variation in their response to different pheromone blends. To address the question of whether differential expression of receptor genes across species is responsible for the ability of males to detect different sex pheromone compounds, the ORs of four closely related species of New Zealand native leafroller moths, *C. obliquana*, *C. herana*, *P. octo* and *P. excessana* were identified. Male antennae often possess a type of sensilla that is either less or not at all present on female antennae (sensilla trichodea), and is associated with pheromone detection. Based on this observation, receptors with higher expression in males compared with females are pheromone receptor (PR) candidates. Using RNAseq-count data and quantitative RT-PCR the differences in the expression of OR genes between males and females were investigated. Identified PR candidates were then functionally tested in HEK293 cell-based calcium assays to verify their function.
To investigate whether amino acid within the OR protein are responsible for differences in the ability of males to detect sex pheromones, the sequence of PR genes and their translated proteins were compared. Pheromone receptors might be under different selection pressures in distinct species depending on whether they are important in the detection of sex pheromones from conspecific females or not. The identified OR genes were tested for positive selection using PAML and predicted protein structures of identified PRs were also compared to find evidence for regions within the protein that might have higher amino substitution rates than others and therefore could be important for sensitivity and specificity of the receptor.
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2.
Ultrastructure of the adult antennae of moths from the genera *Ctenopseustis* and *Planotortrix* (Lepidoptera: Tortricidae)

Bernd Steinwender¹,²,³, Paul Sutherland¹, Richard D. Newcomb¹,²,³

¹ The New Zealand Institute for Plant & Food Research Limited, Auckland, New Zealand
² School of Biological Sciences, University of Auckland, Auckland, New Zealand
³ Allan Wilson Centre for Molecular Ecology and Evolution

**Keywords:**
Antenna, ultrastructure, *Ctenopseustis*, *Planotortrix*, leafroller moth

**Abstract**

The ultrastructure of the antennae of adult male and female moths of four leafroller species was investigated by scanning electron microscopy. *Ctenopseustis obliquana*, *C. herana*, *Planotortrix octo* and *P. excessana* are polyphagous pests of horticulture and endemic to New Zealand. Six types of sensilla were identified on the ventral surface of their antennae: S. trichodea (type I, type II, type III), s. basiconica, s. auricillia, s. chaetica, s. coeloconica and s. styloconica. No major differences in gross antennal morphology or number and type of sensilla were found among the four species. Sexual dimorphism was observed, with sensilla trichodea (type I) only found in males. This finding is consistent with a role for these sensilla in detecting the female sex pheromone.
Introduction

For insects the sense of smell is crucial to orientate within their environment and communicate with other individuals. Moths use chemical cues to locate food sources, oviposition sites and mates (Ache and Young 2005; Stadelbacher et al. 1983). To discriminate between different odorants moths possess olfactory receptors (ORs) (Rutzler and Zwiebel 2005). These ORs are located in sensilla concentrated on their antennae. The antennae of tortricid moths usually consist of three components: scape, pedicel and flagellum (Gomez and Carrasco 2008; Albert and Seabrook 1973; Wall 1978). While the scape and pedicel consist of a single segment each, the flagellum contains several subsegments. The number of these subsegments varies among tortricid moths. In *Talponia batesi* the number of flagellar segments ranges from 34 to 39 (Gomez and Carrasco 2008) and antennae of *Cydia nigricana* possess more than 50 flagellar segments (Wall 1978). Tortricid moths possess a range of sensilla types on their antennae that may be distinguished by their morphological characteristics. Typically there are six different types of sensilla on the antennae of tortricid moths: sensilla trichodea, sensilla basiconica, sensilla auricillia, sensilla chaetica, sensilla coeloconica, sensilla styloconica (Razowski and Wojtusiak 2004). Sensilla trichodea are more abundant on male than on female antennae (Jordan et al. 2008; Koh et al. 1995; Gomez and Carrasco 2008). These s. trichodea contain receptors that bind sex pheromone components produced by females (Rumbo 1981, 1983; Krieger et al. 2005; Krieger et al. 2004). The remaining sensilla are involved in chemo- and mechanoreception (Jung et al. 1999; Wall 1978; den Otter et al. 1978).

Leafroller species within the genera *Ctenopseustis* and *Planotortrix* (Lepidoptera: Tortricidae) are endemic to New Zealand (Dugdale 1990), with sibling species being morphologically cryptic (Dugdale 1990; Wearing et al. 1991). Species range from being highly polyphagous to monophagous on plants such as mangrove or fern (Dugdale 1990). The polyphagous species *Ctenopseustis obliquana, C. herana, Planotortrix octo* and *P. excessana* are considered pests of horticulture in New Zealand (Wearing et al. 1991) and have been investigated more intensively than other species in the two genera. Here we investigate the ultrastructure of adult male and female antennae of these four species by scanning electron microscopy (SEM) and characterise the different types of sensilla present on their antennae.
Materials and Methods

Insects
Insects were reared in the Plant & Food Research insect rearing facility at the Mt Albert Research Centre, Auckland, New Zealand. The history of laboratory strains of *C. obliquana*, *C. herana*, *P. octo* and *P. excessana* has been reported previously (Gleeson et al. 2000). Collected eggs were kept in a humid environment until larvae hatched. Larvae were reared in individual glass tubes containing general purpose diet (Clare and Singh 1988) at 18°C. Adults were kept at 20°C for two days until dissection.

Scanning electron microscopy
Insects were decapitated and dissected heads with antennae were dehydrated in an ethanol series and critically dried in a Baltec CPD 030 critical point drier. Antennae were separated from the heads and samples were mounted onto a carbon sample disc and subsequently attached to scanning electron microscope (SEM) stubs. Silver paint was applied to the proximal end of each antenna to provide improved conductivity. Specimens were sputter coated three times with gold (SEM Coating Unit E5100, Polaron Equipment Ltd, Watford, UK). The ultrastructure of antennae was examined using a FEI Quanta 250 Scanning Electron Microscope (FEI, Hillsboro, USA) at an accelerating voltage of 5 kV.

Types of sensilla were identified following Koh et. al. (1995) and Callahan (1975). The length of sensilla types was measured using ImageJ (Schneider et al. 2012).
Results

The gross morphology of the antenna consists of a single segmented scape and pedicel at the base and multi-subsegmental flagellum extending distally. In both males and females of all four species the number of flagellar subsegments ranged from 44 to 48 (Figure 1). These subsegments consist of morphologically distinct dorsal and ventral surfaces. The dorsal surface, which is exposed when the antennae are held in a resting position, is covered with scales (Figure 2). The ventral surface is characterised by a multitude of hair-like sensilla (Figure 2). This ventral surface faces forward when antennae are held in an alert position. Six distinct types of sensilla were identified on the ventral surface of antenna of all four species; sensilla trichodea, s. basiconica, s. auricillia, s. chaetica, s. coeloconica and s. styloconica.

Figure 1. An antenna of an adult male *Planotortrix octo.*
Sensilla trichodea are on every subsegment and can be divided into three subtypes. Type I sensilla trichodea are present only on male antennae organised in a single row on the proximal end of each segment (Figure 3). Type I s. trichodea were the longest sensilla found on the antennae ranging in size from 86 to 109 µm. Type II s. trichodea are smaller ranging in size from 38 to 76 µm and were found on the antennae of both sexes. The third type of s. trichodea were also found in both males and females and at 27 to 47 µm in length were the shortest of these three types (Figure 4 A-H, Figure 5A). Unlike s. trichodea Type I, Type II and Type III s. trichodea were randomly distributed within each flagellar subsegment. It was difficult to count the absolute numbers of sensilla trichodea on each subsegment. However, an increase in the number of this type of sensilla could be observed. The number of s. trichodea is the lowest on the distal part of the antenna and highest between the 18th and 34th subsegment in both males and females.

Figure 2. Dorsal surface of an antenna from an adult female *Ctenopseustis herana*. Sc, scales.

Figure 3. Subsegment number 30 of a male (left) and female (right) *Planotortrix excessana* antenna. A single row of long sensilla trichodea type I can be observed on the segment of the male but not on females. Tr1, sensilla trichodea type I.
Figure 4. Mean length ± SE of sensilla found on the antennae of A male *Ctenopseustis obliquana*, B female *C. obliquana*, C male *C. herana*, D female *C. herana*, E male *Planotortrix octo*, F female *P. octo*, G male *P. excessana*, H female *P. excessana*. n = 5 per type of sensilla, sex and species.
Figure 5. Sensilla of Ctenopseustis and Planotortrix species A Male Planotortrix excessana showing all three types of sensilla trichodea. One row of s. trichodea type I at the proximal edge of the segment and type II and III. B Close-up of the short sensilla basiconica on a P. octo female antenna. C S. chaetica of a Ctenopseustis herana female. D The shoehorn s. auricillia on the distal edge of a female C. obliquana flagellar segment. E Male P. octo segments with the pit-like s. coeloconica. Tr1, sensilla trichodea type I; Tr2, s. trichodea type II; Tr3, s. trichodea type III; Ba, s. basiconica; Ch, s. chaetica; Au, s. auricillia; Co, s. coeloconica.

Sensilla basiconica range in length from 10 to 22 µm and were distributed randomly across each subsegment. They were found in lower densities compared to the other types of sensilla and were sometimes hard to identify because of their small size (Figure 4 A-H, Figure 5B).
Up to four s. chaetica are located near the centre of each flagellar subsegment and ranged in size from 45 to 84 µm. These sensilla have a socket at their base and several longitudinal grooves that are prominent at the base, becoming more shallow towards the distal end of the sensilla (Figure 5C).

Sensilla auricillia are found at the anterior edges of the subsegment and ranged in size from 15 to 33 µm. These bladelike sensilla have longitudinal grooves giving them a concaved shape (Figure 5D).

Sensilla coeloconica consist of a circular pit-like structure with one sensillum in the centre of the pit protected by 10 – 15 short sensilla around the edges. The length of these short sensilla ranged from 4 to 10 µm and the diameter of the pit ranged from 6 to 10 µm. They are found clumped together mostly on the anterior edge of the flagellar subsegment (Figure 5E).

There is usually one sensillum styloconica per flagellar subsegment located on the ventral anterior surface of the subsegment, except for the distal end of the antenna where there are more than one sensillum per subsegment (Figure 6A-D and 7A). In general these sensilla are phallic structures with the sensilla protruding from a sheath. These sensilla differed in size and shape falling into three different subtypes with long sensilla at the tip, the most common medium sized sensilla that were present on every segment, ranging in length from 16 to 29 µm and a very short version that is typically found on the edges of the segments (Figure 6B).
Figure 6. Different shapes of sensilla styloconica on the distal tip of antennae from different individuals. A Female *Ctenopseustis herana*. B Male *C. obliquana*. C Female *Planotortrix excessana*. D Female *P. octo*. St, s. styloconica.

Figure 7. Sensilla styloconicum of *Ctenopseustis* and *Planotortrix* species A s. styloconicum on the antennal subsegment of a female *Planotortrix excessana*. B Short s. styloconicum on an antennal segment of a female *Ctenopseustis obliquana*. St, sensillum styloconicum.
Discussion

We have investigated the ultrastructure of adult male and female antennae of the leafroller moths *C. obliquana, C. herana, P. octo* and *P. excessana* using scanning electron microscopy. The antennal morphology in all four investigated species was similar, except for the two *Planotortrix* species having slightly longer sensilla than *Ctenopseustis* species due to their large size. The most recent common ancestor within *Ctenopseustis* species is estimated to be less than 500 000 years ago and within *Planotortrix* even more recently (Langhoff et al. 2009) and it is still possible to produce fertile hybrids within the genus (Foster et al. 1997; Hansson et al. 1989; Albre et al. 2013). The very young age of these species likely explains the similar setup of segments and sensilla among the four species.

The overall antennal morphology is similar to other lepidopteran species with the scape and pedicel on the base and the flagellum consisting of multiple subsegments (Jordan et al. 2008; Koh et al. 1995).

Furthermore, as found in the closely related species, *Epiphyas postvittana*, the dorsal surface of the antennae is covered in scales (Jordan et al. 2008). The scales are thought to protect the antennae and fragile sensilla from mechanical damage (Koh et al. 1995), increase an individual’s ability to detect stimulus direction (van der Pers et al. 1980) and/or to concentrate odour molecules (Wall 1978). The ventral surface is covered with several types of sensilla. The number of different types of sensilla identified matched with other tortricids like *E. postvittana* (Jordan et al. 2008), *T. batesi* (Gomez and Carrasco 2008) or *H. assulta* (Koh et al. 1995). The largest morphological differences to the closely related species *E. postvittana* was in the position and arrangement of the s. trichodea type I, important for sex pheromone reception, and in the shape of s. styloconica.

In total six different types of sensilla were identified on the ventral surface: sensilla trichodea, sensilla basiconica, sensilla auricillia, sensilla chaetica, sensilla coeloconica and sensilla styloconica. These types of sensilla were similar to those found in the tortricid moth *E. postvittana* (Jordan et al. 2008) and *Cydia nigricana* (Wall 1978). In *C. nigricana* an additional type sensilla was identified, sensilla squamiformia, that is missing from the species investigated here (Wall 1978). In *Talponia batesi* all types of sensilla were identified except for sensilla basiconica (Gomez and Carrasco 2008).

In all four species investigated here there was sexual dimorphism in the ultrastructure of the antennae. Males in these New Zealand endemic leafroller species possess one radial row of long sensilla trichodea type I that was not present in female antennae. In the closely related *E. postvittana* the long sensilla trichodea type I was present in two rows (Jordan et al. 2008), in *H. assulta* sensilla trichodea type I were found in 3 – 4 circumferential rows (Koh et al. 1995) and in *T. batesi* male biased s. trichodea were randomly distributed within the segments (Gomez and Carrasco 2008). These sensilla trichodea type I are involved in sex pheromone detection in moths (Rumbo 1981, 1983; Krieger et al.
2005; den Otter et al. 1978; Hansson et al. 1989) and are usually innervated by one to three neurons (Keil 1999). Electrophysiological investigations in two species of the genus Ctenopseustis, C. obliquana and C. herana, showed that males in these species possess s. trichodea with two neurons, a large spike amplitude cell that responds strongly to the main pheromone component and a small spike amplitude cell that responds weakly to the minor component (Hansson et al. 1989). While the long s. trichodea type I was only present in males, all other sensilla were present in both sexes.

The short s. basiconicum showed dorsal flattening, were found randomly dispersed across the ventral surface of the flagellar subsegment and were not present on the more distal segments of the antenna. This type of sensilla is thought to detect host plant volatiles (Sun et al. 2011) and in Spodoptera littoralis to respond to green leaf volatiles and flower (Anderson et al. 1995).

The shoehorn shaped s. auricillium were found at the distal edge of the ventral surface of each subsegment. The position of these sensilla within the subsegment is consistent with other species including Helicoverpa assulta (Koh et al. 1995), T. batesi (Gomez and Carrasco 2008), Ostrinia nubilalis (Cornford et al. 1973) and C. nigricana (Wall 1978) and are thought to respond to plant volatiles (Wall 1978; den Otter et al. 1978).

S. chaetica possess a socket on the base of the sensilla that is also found in other tortricid moths (Gomez and Carrasco 2008; Wall 1978). Four sensilla are present per segment on the ventral surface. In T. batesi these types of sensilla are also found on the dorsal surface of the flagellar segments (Gomez and Carrasco 2008) and also in O. furnacalis (Jung et al. 1999), which is not the case in any of the species investigated here. It has been suggested that s. chaetica function as a combination of contact chemoreceptor and mechanoreceptor (Albert and Seabrook 1973; Razowski and Wojtusiak 2004).

The pit-like sensilla coeloconica were found mainly on the distal edge of the ventral flagellar segments and occasionally in the centre of the segment. Only one type of s. coeloconica could be identified in Ctenopseustis obliquana, C. herana, Planotortrix octo and P. excessana. This finding is congruent with many other species of tortricid moths (Gomez and Carrasco 2008; Albert and Seabrook 1973; Callahan 1975) but distinct from C. nigricana where two types were identified (Wall 1978). Sensilla coeloconica in Bombyx mori respond to acids and aldehydes (Pophof 1997).

Different shapes and sizes of sensilla styloconica could be identified in the New Zealand endemic leaf roller moths. Even though the general appearance and position of these sensilla was similar to other species such as T. batesi or H. assulta (Gomez and Carrasco 2008; Koh et al. 1995), the shape was variable between individuals especially at the distal end of the antenna (Figure 4A-D). Sensilla styloconica are thought to be thermo- and hygroreceptors (Hallberg 1981) or chemoreceptors (Wall 1978).
In summary the sensilla on the antennae of these four *Ctenopseustis* and *Planotortrix* species contain six sensilla types and display sexual dimorphism in their long sensilla trichodea type I consistent with a role on sex pheromone detection. This work paves the way for the use of differential expression analysis of odorant receptor genes to identify candidate receptors in males that may respond to sex pheromone compounds produced by females.
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References


Pheromone receptor evolution in the cryptic leafroller species, *Ctenopseustis obliquana* and *C. herana*

Bernd Steinwender1, Amali H. Thrimawithana1, Ross Crowhurst1, Richard D. Newcomb1,2,3

1 The New Zealand Institute for Plant & Food Research Limited, 120 Mt Albert Road, Sandringham, Auckland, 1025, New Zealand
2 School of Biological Sciences, University of Auckland, 3a Symonds Street, Auckland Central, Auckland, 1010, New Zealand,
3 Allan Wilson Centre for Molecular Ecology and Evolution

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Abstract

How new mate recognition systems evolve when changes are required in both the male and female components remains a conundrum. Here we investigated the molecular basis of pheromone reception in two closely-related species of tortricid (leafroller) moth, *Ctenopseustis obliquana* and *C. herana*. Male *C. obliquana* are attracted to a 90:10 blend of (Z)-8-tetradecenyl acetate (Z8-14:OAc) and (Z)-5-tetradecenyl acetate (Z5-14:OAc), while *C. herana* males are attracted to Z5-14:OAc alone. We used a transcriptome sequencing approach from adult male and female antennae to identify 47 olfactory receptors (ORs) from each species and assessed their expression levels in male and female antennae using RNA Seq-counting and quantitative RT-PCR. Three male biased and one female biased OR were identified in *C. obliquana* by quantitative RT-PCR, and four male biased and one female biased receptor in *C. herana*. The male biased receptors, CoblOR7, CoblOR30, CherOR7, CherOR30, CherOR1a and CherOR1b were tested for their ability to respond to sex pheromone components in a HEK293 cell calcium assay. CoblOR7 and CherOR7 responded to Z8-14:OAc, however no receptor for Z5-14:OAc, the component used by both species, was identified. Interestingly CherOR7 has reduced sensitivity to Z8-14:OAc and unlike CoblOR7, also responds to Z7-14:OAc, indicating that CherOR7 may be under relaxed constraint compared with CoblOR7. Significantly higher sequence differences were found in the third and the sixth transmembrane domain region and PAML analysis indicated positive selection acting on CoblOR7. These findings are consistent with electrophysiological data revealing the presence of neurons tuned to both Z8-14:OAc and Z5-14:OAc in both species, but that for *C. herana* males, the ability to specifically detect Z8-14:OAc is currently not required.
Introduction

Changes in both the female and the male components are required for new mating systems to evolve. Pheromone-based mating systems offer a discrete communication system to understand how such systems evolve and are becoming increasingly studied for this purpose (Niehuis et al. 2013; Smadja and Butlin 2009; Shirangi et al. 2009; Symonds and Elgar 2008; Albre et al. 2013; Lassance and Lofstedt 2009). In moths, males locate females through upwind flight along a concentration gradient of the sex pheromone (Bradbury and Vehrencamp 1998). Compounds that make up the sex pheromone are mainly fatty-acid derivatives such as acetates, alcohols, and aldehydes that are typically 10, 12, 14, 16 or 18 carbons in length with one or two unsaturated positions along the chain (Linn and Roelofs 1995). Typically pheromone blends are composed of one major component and one or more minor components. The composition of those sex pheromone components is often highly specific, with little variation within populations. Species specificity in pheromone production and reception forms a robust mate recognition system that limits incompatible mating events (Linn and Roelofs 1995).

How new sex pheromones and pheromone blends are biosynthesised has been investigated in several species of moths, revealing changes in both desaturases or fatty acid reductases (Shirangi et al. 2009; Greenberg et al. 2003; Sakai et al. 2009; Albre et al. 2012; Xue et al. 2007). Sex pheromones are produced from simple fatty acids in specialised glands in the abdomen of females where the components are synthesised. Double-bonds are introduced into fatty acids through specialized fatty-acyl desaturases (Knipple et al. 2002), often with several desaturases present possessing distinct affinities for different fatty acids substrates (Lienard et al. 2008). Differential expression of the genes encoding these desaturase enzymes can be crucial for producing new pheromone components. In the Ostrinia species, O. nubilalis and O. furnacalis, for example, the activation of ancestral desaturase genes, together with gene duplication and retroposon fusion has produced novel desaturase activities (Roelofs et al. 2002; Roelofs and Rooney 2003; Xue et al. 2007; Fujii et al. 2011). In O. scapulalis and O. furnacalis the differential expression of desaturase genes is responsible for the production of species-specific pheromone components (Sakai et al. 2009). In addition to desaturases, altered fatty-acyl reductases (FARs) can produce distinct pheromones through differences in affinities for desaturased precursors in their conversion from fatty acids to alcohols. For example, the pheromones of the E and Z strains of O. nubilalis are produced by distinct alleles of the pheromone FAR. The alleles differ in the substrate specificity which leads to differentially reduced ratios in the final pheromone blend (Lassance et al. 2010).
How new pheromones are derived has been studied extensively but to understand the evolution of mating systems as a whole it is necessary to understand the role of the receiver and how the evolution of sex pheromone perception proceeds especially at the molecular level (Symonds and Elgar 2008). It seems difficult for mating systems that rely on chemical cues to evolve rapidly because purifying selection should prevent changes in either pheromone production or perception. A hypothesis that attempts to solve this dilemma is the “asymmetric tracking hypothesis”, which suggests that in a species where females are the limiting sex, greater variation may be tolerated in the male’s pheromone perception system to provide a scenario where rare males may exist that are able to sense a novel pheromone blend (Phelan 1992; Domingue et al. 2007). In males changes in the preference for certain pheromones may well depend on alterations in a multigene family of receptors responsible for detecting the sex pheromone components (Gould et al. 2010; Wanner et al. 2010; Miura et al. 2010; Leary et al. 2012). It has been shown that even single mutations in the sequence of olfactory receptors OR genes can change the specificity of the receptor protein (Leary et al. 2012).

Moths perceive odorants through olfactory receptors (ORs) located within sensilla on their antennae. These ORs contain seven transmembrane regions with a cytoplasmic N terminus and an external C terminus (Smart et al. 2008; Benton et al. 2006; Lundin et al. 2007). Together with the olfactory receptor co-receptor (Orco), they form a ligand-gated cation channel. While Orco is highly conserved (Guo and Kim 2007), ligand-binding ORs are more variable, with the C terminus being more highly conserved than the N terminus (Tunstall et al. 2007; Corcoran 2014). ORs involved in sex pheromone perception in moths are located in specialized long hair-like sensilla called sensilla trichodea (Rumbo 1981, 1983). These very long sensilla are typically more abundant on male antennae compared with females (Heinbockel and Kaissling 1996; Jordan et al. 2008; Mitsuno et al. 2008; Sakurai et al. 2004; Krieger et al. 2004). Receptors associated with sex pheromone perception in moths to date all fall into a separate phylogenetic clade and typically show higher levels of gene expression in the antennae of males compared with females.

Many olfactory receptors and pheromone receptors have been identified from a handful of moth species using a range of techniques including whole genome sequencing and RNA seq. From Heliothis virescens 21 candidate OR genes have been identified in whole genome assemblies by BLAST searches using Drosophila ORs (Krieger et al. 2002). Forty nine ORs were identified in the silkworm, Bombyx mori (Anderson et al. 2009), 43 in the coding moth, Cydia pomonella (Bengtsson et al. 2012), 47 in the tobacco hornworm, Manduca sexta (Grosse-Wilde et al. 2011) and 70 in the light-brown apple moth, Epiphyas postvittana (Corcoran 2014). From these and a range of other moths, sex pheromone receptors (PRs) have been identified, initially through evidence of male biased expression or phylogenetic position and then through functional studies in a variety of assay systems, including HEK293 cells (Grosse-Wilde et al. 2007; Forstner et al. 2009), Xenopus oocytes (Sakurai et al. 2004; Nakagawa et al. 2005; Mitsuno et al. 2008; Miura et al. 2010; Wang et al. 2011; Wanner et
al. 2010) and also in *Drosophila* olfactory receptor neurons (Syed et al. 2006; Kurtovic et al. 2007; Montagne et al. 2012).

The endemic New Zealand genera, *Ctenopseustis* (brown-headed leafroller, five species) and *Planotortrix* (green headed leafroller, seven species) (Tortricidae: Lepidoptera) include widespread and highly localised species (Dugdale 1990; Newcomb et al. 2014 in press). Adult wing patterning is highly variable within and between species, making many of the species within a genus morphologically cryptic (Dugdale 1990; Wearing et al. 1991). Many of the speciation events are thought to be very recent within the last million years, with some species not being able to be resolved using variation in neutral molecular markers due to incomplete lineage sorting (Langhoff et al. 2009). Within the two genera there are examples of highly polyphagus and some monophagous species on plants such as mangrove and fern. The polyphagous species *C. obliquana*, *C. herana*, *P. octo* and *P. excessana* together with the light-brown apple moth, *Epiphyas postvittana*, form a complex of horticultural pests in New Zealand (Wearing et al. 1991) and therefore have received more attention than the other species in *Ctenopseustis* and *Planotortrix* in terms of research.

Females of the two genera produce sex pheromone blends consisting of tetradecenyl acetates unsaturated at either of the 5, 7, 8 or 9 positions in the cis (Z) conformation (Roelofs and Brown 1982; Newcomb and Gleeson 1998; Foster et al. 1986). These double bonds are introduced through specialised fatty-acyl desaturases found in female pheromone glands (Foster and Roelofs 1988, 1996). Recently studies have been undertaken to investigate the regulation of desaturase genes in the pest species (Albre et al. 2012; Albre et al. 2013). Expression levels a Δ10-desaturase of *C. obliquana* and *C. herana* as well as *P. octo* and *P. excessana* are concordant with the presence (*C. obliquana* and *P. octo*) or absence (*C. herana* and *P. excessana*) of the pheromone component (Z)-8-tetradecenyl acetate (Z8-14:OAc) in the blend of respective species (Albre et al. 2012). Further crossing experiments conducted in the lab between *P. octo* and *P. excessana* revealed that the difference in expression of *desat5*, which encodes the Δ10-desaturase, is controlled by a trans-acting repressor and requires a cis-regulatory mutation in the *desat5* promoter (Albre et al. 2013).

As well as sex pheromone production, the male’s ability to perceive pheromone compounds has also been investigated in the closely related species, *C. obliquana* and *C. herana*. These species have overlapping distributions with *C. obliquana* found throughout New Zealand and *C. herana* restricted to the South Island. While *C. obliquana* males are attracted to a 90:10 blend of Z8-14:OAc and (Z)-5-tetradecenyl acetate (Z5-14:OAc) (Foster et al. 1986; Young et al. 1985), *C. herana* males are attracted to Z5-14:OAc alone (Foster and Roelofs 1987). Electrophysiological studies showed that trichoid sensilla of *C. obliquana* contain a large spike amplitude cell that responds strongly to the main component, Z8-14:OAc, and a small spike amplitude cell responding to the minor component, Z5-14:OAc. In *C. herana* the opposite is the case, with the large spike amplitude cell responding to
Z5-14:OAc and a small spike amplitude cell responding to Z8-14:OAc (Hansson et al. 1989). In wind
tunnel experiments males of each species responded very selectively to pheromone blends produced
by con-specific females and only a few males were attracted by blends deviating from those produced
by pure bred females (Foster et al. 1997). Field cage experiments showed that males of both species
are only attracted to con-specific females (Clearwater et al. 1991) and genetic analysis provide
evidence for no interbreeding in the wild between the two species (Langhoff et al. 2009).

Here we produce transcriptome databases constructed from the antennae of male and female *C.
obliquana* and *C. herana* and mine them for odorant receptors. In the New Zealand native leafroller
moths antennae show a strong sexual dimorphism with male antennae possessing an abundance of
sensilla trichoidea type I, whereas females possess none (Chapter 2). We identify candidate
pheromone receptors through phylogenetic analysis and through the determination of differences in
the levels of gene expression between male and female antennae. From a short list of candidate
receptors, functional studies in HEK293 cells identify receptors that respond to pheromone.
Material and Methods

Insects

Insects were reared in the Plant & Food Research insect rearing facility at the Mt Albert Research Centre, Auckland, New Zealand. The history of the lab strains of *C. obliquana* and *C. herana* has been reported previously (Gleeson et al. 2000). Eggs were collected and kept in a humid environment until larvae hatched. Larvae were reared separately in small glass tubes containing a general purpose diet (Clare and Singh 1988) at 18°C. Pupae and adults were kept at 20°C on a 16:8 light cycle. Adult moths were provided with cotton cloth soaked in water.

Nucleic acid isolation

RNA for transcriptome sequencing was isolated from 100 male and female antennae each dissected from 2-3 days old adults. RNA was extracted and purified using 800 µl Trizol (Invitrogen, Carlsbad, CA, USA) following the TRizol Plus RNA Purification Kit protocol. DNase treatment was conducted on 10 µg of total RNA using the TURBO DNA-free Kit (Life technologies, Carlsbad, CA, USA) following the manufacturer’s instructions on antennal RNA of *C. herana*.

RNA for quantitative RT-PCR (qPCR) experiments was isolated from male and female antennae dissected from 2-3 days old individuals, as well as whole bodies, using 800 µl Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. The initial screening for male-biased expression of odorant receptors was conducted with pools of ten antennae pairs; however for subsequent qPCR experiments RNA extracted from single antennae pairs was used. One µg of extracted RNA was treated with DNase (DNaseI amplification grade, Invitrogen) following the manufacturer’s instructions. cDNA was synthesised using iScript cDNA Synthesis Kit (Bio-Rad) from 1 µg of total RNA, incubated at 25°C for 5 minutes, 42°C for 30 minutes and 85°C for 5 minutes.

Next-generation sequencing and bioinformatics

RNAseq libraries were constructed from both male and female adult antennae of *C. obliquana* and *C. herana* using Illumina’s standard protocols and sequenced at Macrogen (Seoul, South Korea). Quality score analysis on the read pairs for each library was undertaken using FastQC (FastQC 2008). In-house Perl scripts were used to trim all reads by 13 bases at their 5' ends and remove any read pairs containing Ns and mononucleotides. Mitochondrial contamination was removed by mapping RNASeq read pairs to a reference mitochondrial genome of *C. obliquana* assembled from a draft genome. Mapping was performed using bowtie (version 1.0.0) (Langmead et al. 2009) with the reads mapping to the mitochondrial genome being removed. Thereafter, read pairs were trimmed to a minimum quality threshold of 20 using fastq-mcf from the ea-utils package (Aronesty 2011). Duplicates within the read files for *C. obliquana* were removed using in-house Perl scripts prior to assembly, while the
redundancy in the *C. herana* was removed after the assembly using cd-hit (Li and Godzik 2006). De novo assembly of the processed reads was performed for each of the individual libraries (Supplementary Material 1) with trans-ABYSS (version 1.3.2) (Robertson et al. 2010), where a k-mer series of 31 to 75 with an increment of two bases was used for the libraries of *C. obliquana*, whereas k-mer values from 31 to 71 were used for the *C. herana* libraries.

Candidate ORs were identified using tblastn (Altschul et al. 1990) with the amino acid sequences of 70 ORs from the leafroller moth *Epiphyas postvittana* (Corcoran 2014) used as queries. Full length open reading frames of ORs were acquired using tblastn against the assembled contigs of male and female antennae from *C. obliquana* and *C. herana*. Where necessary, a draft genome assembly of *C. obliquana* was used to extend sequences (unpublished data). The post-processed RNASeq reads were mapped onto a constructed set of OR sequences using bowtie (version 2.1.0) (Langmead and Salzberg 2012). The resulting alignment was then used to obtain expected read counts using multBamCov from the bedtools package (v2.16.2) (Quinlan and Hall 2010) and cufflinks (v2.1.1) (Trapnell et al. 2010) for Fragments Per Kilobase of transcript per Million reads (FPKM).

Sequence data was edited and aligned in Geneious (Kearse et al. 2012) using ClustalW with *E. postvittana* sequences. Phlogenetic trees were generated in Mega (Hall 2013) with a model chosen by ModelTest (Posada and Crandall 1998). The dN and dS rates were estimated using codon-based substitution models in PAML version 4.7 (Yang 2007) by using M0, with one fixed ω ratio, and M3 (Yang and Nielsen 2000), which has three categories of site with a free ω ratio for each site class. Transmembrane domains were predicted using SPLIT 4.0 (Juretic et al. 2002) at the transmembrane prediction server (http://split4.pmfst.hr/split/4/). The topology diagram was constructed using TOPO2 Transmembrane Protein Display (Johs 2005) by the server at (http://www.sacs.ucsf.edu/TOPO2).

**Quantitative Real-Time PCR**

The expression levels of ORs in male and female antennae, as well as bodies, were determined by quantitative real-time PCR relative to *α*-tubulin, *β*-actin and elongation factor 1α (Turner et al. 2006). Primers were designed to the *C. obliquana* receptors and tested on the orthologous receptors from the *C. herana* (Supplementary Material 2) and genomic DNA in both species. Reactions (10 µL) included 20 ng of cDNA, 5 µL 2x SYBR Green Mix (Bio-Rad), and 200 nM of each forward and reverse primer. Quantitative real-time PCR cycling conditions were set up as following, 2 minutes at 95°C followed by 45 cycles of 15 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C. A final dissociation curve analysis was added with 15 seconds at 95°C, 15 seconds at 60°C and a gradual heating to 95°C at 0.01°C/s. Experiments were carried out with three biological replicates and three technical replicates per biological replicate, with negative controls for each replicate. Thermocycling was conducted on a LightCycler480 Real-Time instrument (Roche Diagnostics, Basel, Switzerland).
To determine the amplification efficiency and the cycle threshold values for each reaction the software LinRegPCRv11 was used (Ramakers et al. 2003). The relative expression levels were calculated following a modified version of the ΔCp method (Pfaffl 2001; Livak and Schmittgen 2001; Albre et al. 2013) as described in Corcoran et al (2014a).

**Cell-based assays**

Primers were designed to the 5' and 3' ends of the predicted open reading frames of ORs that showed male-biased expression in antennae or were members of the so-called pheromone receptor clade (Supplementary Material 3 for primer sequences). Standard PCR amplifications were carried out in 50 µL reaction volumes containing 0.5 U Platinum Taq polymerase (Invitrogen), 1 x reaction buffer, 1.25 mM magnesium chloride, 0.2 mM dNTP mix, and 0.2 µM of each primer, with 2 µL of cDNA as template. For the PCR amplifications a GeneAmp 9700 (Applied Biosystems) PCR machine was used with an initial denaturation step of 5 min at 94°C, followed by 35 cycles (94°C for 10 seconds, 56°C for 30 seconds, 72°C for 45 seconds – 1.5 minutes), and a final elongation step at 72°C for 7 minutes. PCR products were resolved on a 0.7 % TAE gel at 80 V for 60 minutes. Gel pieces were extracted using the QIAquick Gel Extraction Kit (QIAGEN). Extracted PCR products were cloned into pCR8/GW/TOPO vectors (Invitrogen) and sequenced at Macrogen, Seoul, South Korea, using M13 forward and reverse primers. Once at least two clones of identical sequence were identified those plasmids were used for cloning into the expression vector. Full length clones were used as template to amplify full length sequences containing restriction sites for transformation into the pcDNA 5/TO expression vector (Invitrogen) (see Supplementary Material 4 for primer sequences). Again several clones were sequenced to check that acquired genes contained the correct sequence. Candidate genes were transfected into isogenic TREx HEK293 cell lines containing the *E. postvittana* odorant receptor co-receptor, EposOrco, which has 99.6 % amino-acid identity to the Orco orthologue in both *Ctenopseustis* species, following the protocol described in Corcoran 2014 except that no single-cell sorting was conducted. Functional assays were carried out as described in Corcoran 2014. Briefly, 25,000 cells were plated into each well of a poly-d-lysine-coated, black-walled 96-well cell culture plate (Becton Dickenson) and grown overnight (37°C, 5% CO₂). The next day the cell culture medium was replaced with fresh medium with 1 µg/ml doxycycline for induction and without doxycycline as control. Cells were grown again under above conditions for sixteen to twenty four hours before functional testing. Before the assay the cell culture medium was removed and rinsed with assay buffer (DPBS containing 1 mM probenicid, pH 7.1). Fifty µL of loading buffer (assay buffer containing 1µM Fluor4-AM (Life Technologies) and 0.2% pluronic acid) was added to each well and incubated for 30 minutes at room temperature in the dark. Then wells were rinsed twice with assay buffer, 100 µl of assay buffer was added followed by incubation for another 30 minutes at room temperature in the dark. Cell assays were executed on an Omega FluoStar plate reader system. Baseline fluorescence was determined by exciting wells at 485 nM and reading resulting fluorescence at 535 nM. Receptors
were screened with 30 µM mono-unsaturated tetradecenylacetates (in assay buffer with 0.5 % DMSO) that are used most commonly as sex-pheromones in the New Zealand endemic leafroller moth genera *Ctenopseustis* and *Planotortrix* (Newcomb and Gleeson 1998), including saturated tetradecenyl acetate (14:OAc), (Z)-5-tetradecenyl acetate (Z5-14:OAc), (Z)-7-tetradecenyl acetate (Z7-14:OAc) and (Z)-8-tetradecenyl acetate (Z8-14:OAc) (Pherobank, Netherlands; 95 % to 99% purity). As a vehicle control 0.5% DMSO in assay buffer and as a control for Orco expression 50 µM of the insect Orco agonist VUAA1 (Jones et al. 2011) were used. After baseline determination, compounds were added to three non-induced and three induced wells. Change in fluorescence was measured for a period of 60 seconds after addition of the compound, vehicle control or VUAA1.

Experiments for concentration-response curves for compound-receptor combinations where positive responses were observed followed the same protocol for preparing plates and cells as for the screening experiments. In concentration-response experiments increasing concentrations from 0.014 µM up to 300 µM of compound were used for the compounds Z7-14:OAc and Z8-14:OAc in CoblOR7 and CherOR7. The mean response (+/- SEM) from each three of induced and non-induced wells were used to construct concentration-response curves using the non-linear regression function of GraphPad data analysis software (GraphPad, Inc).
Fig. 1 Phylogenetic analyses of odorant receptors from *Ctenopseustis obliquana* and *C. herana* A Circle tree of odorant receptors from *C. obliquana* and *C. herana*, together with those from *Epiphyas postvittana*. The tree is rooted with the odorant receptor co-receptor, Orco. The positions of OR01, OR07 and OR30 are indicated with arrows. The sex-pheromone receptor clade is highlighted with a semi-circle. The position of singleton receptors CoblOR37 and CherOR66 are indicated with arrows B Phylogeny of the sex pheromone receptor clade containing odorant receptor genes of *E. postvittana*, *C. obliquana* and *C. herana*. Odorant receptors displaying higher levels of expression in adult male compared with female antennae are highlighted. These include OR07 and OR01, showing the two copies of OR01 in *C. herana*. C Phylogeny of the second OR clade containing receptors from *E. postvittana*, *C. obliquana* and *C. herana*. Odorant receptors displaying higher levels of expression in adult male compared with female antennae.
Results

Four RNAseq libraries were generated from the antennae of adult male and female *C. obliquana* and *C. herana*, generating from 39,823,878 to 71,083,823 raw sequence reads (Supplementary Material 5). RNAseq data has been deposited into a Sequence Read Archive (SRA) database online (http://www.ncbi.nlm.nih.gov/) under the accession numbers 236626 and 236627. Resulting assemblies ranged in the number of contigs from 102,817 to 226,501 (Supplementary Material 1). Differences between species in the numbers of contigs may be associated with differences in DNAse treatment of RNA and/or removal of duplicates at different stages of the filtering process. Blast sets were established for the four transcriptomes and queried using the 70 ORs available from *E. postvittana* (Corcoran 2014). A draft genome of *C. obliquana* (unpublished data) was also employed to find additional sequences of ORs if missing from the antennal transcriptome assembles. Using these resources transcripts of 47 OR genes from *C. obliquana* and 47 from *C. herana* were identified in antennae and predicted protein sequences derived and the numbering of identified receptors follows largely the numbering of the *E. postvittana* orthologos. Except for one OR from outside the sex pheromone receptor clade of each species (CoblOR66 and CherOR37), all receptors were represented by orthologous pairs (Fig. 1a). The orthologous ORs share from 89% to 99% amino-acid identity (Supplementary Material 6). The clade predicted to contain the sex pheromone receptors of many moth species is well supported by bootstrap analysis (94% from 1000 bootstrap replicates). However, orthology among the ORs of the two *Ctenopseustis* species and *E. postvittana* within this pheromone receptor clade is less clear (Fig. 1b), unlike other receptors that show male biased expression from outside this clade in *E. postvittana* and the *Ctenopseustis* species (see later; Fig. 1c).

Gene expression of ORs

Because in moths sex pheromone receptors are often more highly expressed in male than in female antennae (Krieger et al. 2004; Grosse-Wilde et al. 2010), we identified receptors that showed this pattern of sexual dimorphic expression between the sexes in antennae. Using RNA seq-counting with a two-fold cut-off criteria, six OR genes were identified as having male biased expression in both species. Two ORs were identified as male biased in both species, OR7 and OR30, while in *C. herana* two similar genes (CherOR1a and CherOR1b; 86.9 % identical at the amino acid level), also showed male biased expression. CherOR1b is homologous to CoblOR1 in *C. obliquana*, whereas no related gene was found for OR01a in *C. obliquana*. Of the ORs that show male biased expression, OR01 and OR07 are located in the sex pheromone receptor clade, while OR30 is located outside this clade in the phylogeny (Fig. 1a). RNA-seq count also revealed ORs that are more highly expressed in female than in male antennae (Supplementary Material 7 and 8). With a two-fold cut-off on RNA-seq count also revealed six female biased ORs in *C. obliquana* (CoblOR14, CoblOR22, CoblOR25, CoblOR49,
CoblOR58 and CoblOR63) and nine in C. herana (CherOR12, CherOR22, CherOR25, CherOR45, CherOR49, CherOR52, CherOR58, CherOR63 and CherOR64).

Thirty two genes in C. obliquana and 21 in C. herana were assessed for their levels of gene expression using qPCR. These included the three male biased receptors and other four members from the pheromone receptor clade of C. obliquana and four male biased receptors and the remaining four in the pheromone receptor clade of C. herana. Of those tested three OR genes were significantly male biased in their expression in the antennae of both species (Supplementary Material 9 and 10). As found with RNA-seq count, OR7 and OR30 were significantly male biased (CoblOR7 \( P = 0.044 \); CherOR7 \( P = 0.014 \); CoblOR30 \( P = 0.002 \); CherOR30 \( P = 0.005 \)), as was OR1 (CoblOR1 \( P = 0.004 \); CherOR1 \( P = 0.005 \)) (Fig. 2). However, it should be noted that the primers designed for the qPCR screening for the two versions of OR1 in C. herana were located in regions were the paralogous genes are very similar and therefore the results for the two OR1 genes from C. herana may be confounded. Attempts to design primers in regions that are distinct in the two genes failed to result in primers useful for qPCR. In C. obliquana where there is only one version of OR1, the transcript could be detected as male biased by qPCR. Quantitative PCR failed to confirm female biased expression of tested receptors from C. obliquana (CoblOR14 \( P = 0.119 \), CoblOR22 \( P = 0.098 \), CoblOR25 \( P = 0.263 \), CoblOR49 \( P = 0.140 \)) and C. herana (CherOR12 \( P = 0.315 \), CherOR22 \( P = 0.110 \), CherOR25 \( P = 0.565 \)). Overall, of the 13 receptors that were identified by RNA-seq as potentially male or female biased and successfully tested in qPCR experiments, six were confirmed to show sex biased gene expression. All of the male biased ORs were significantly more highly expressed when tested in qPCR and potentially female biased ORs showed a trend in this direction, although no receptor was statistically significant. This suggests that use of RNA-seq is a reasonable method to identify candidate OR genes for further testing.

**Fig. 2** Relative expression of the odorant receptors OR01, OR07 and OR30 in the male (blue) and female (red) antennae of adult **A** Ctenopsueustis obliquana and **B** C. herana. CT values are the means ± SE normalised to the housekeeping genes α-tubulin, β-actin and elongation factor 1α (BDL = below limits of detection)
**Cell assays**

The full length cDNA sequences of three receptors from *C. obliquana* (OR1, OR7 and OR30) and their orthologues from *C. herana*, plus one additional OR (CherOR1a) were recovered by PCR from antennal cDNA. These seven OR genes were cloned into expression vectors, confirmed by sequencing and tested in cell assays against four mono-unsaturated tetradecenyl acetates. Of the receptors tested only OR7 from *C. obliquana* and *C. herana* responded to at least one compound tested. While CoblOR7 responded to Z8-14:OAc (EC$_{50}$ = 651±33 nM) alone (Fig. 3a and 3b), CherOR7 responded to Z7-14:OAc (EC$_{50}$ = 2012±32 nM) and Z8-14:OAc (EC$_{50}$ = 4022±30 nM) (Fig. 3c and 3d). The remaining receptors showed no response to any of the tested components, with no receptor found that responded to Z5-14:OAc. It cannot be ruled out that these receptors (CoblOR1, CoblOR30, CherOR1a, CherOR1b, CherOR30) are capable of responding to pheromone components but are just not properly expressed or trafficked to the membrane in HEK293 cells.

**Fig. 3** Concentration-response curves of OR07 from *Ctenopseustis obliquana* and *C. herana* three induced wells (red) and three un-induced wells (blue) 10 seconds after injection of the test compound. Each data point represents the % increase in fluorescence at a certain concentration. A CoblOR07 response to (Z)-7-tetradecenyl acetate B CoblOR07 response to (Z)-8-tetradecenyl acetate C CherOR07 response to (Z)-7-tetradecenyl acetate D CherOR07 response to (Z)-8-tetradecenyl acetate

**Sequence comparison of CoblOR7 and CherOR7**

A likelihood ratio test to find evidence for positive selection in M0 vs M3 was significant ($P = 0.007$; $2\Delta l = 17.77$) indicating positive selection acting on CoblOR7. A comparison with orthologous genes of OR7 in the sister genus *Planotortrix* (data not shown) reveals a dN/dS ratio of $\omega = 0.963$ for *C. obliquana* (29.1 non-synonymous and 10.9 synonymous changes) and for *C. herana* $\omega = 0.1311$ (1.8
non-synonymous and 5.1 synonymous changes) (Fig. 4a). Both, the likelihood ration test and the elevated dN/dS ratio in CoblOR7 compared to its orthologue CherOR7 indicate possible positive selection acting on CoblOR7.

To identify potential substitutions involved in encoding the selectivity differences between CoblOR7 and CherOR7, as well as to investigate evidence for relaxed constraint we compared the sequence of the two receptors. CoblOR7 and CherOR7 differ by 45 nucleotide substitutions, comprising 13 synonymous and 29 non-synonymous substitutions. The 29 non-synonymous substitutions are distributed across the predicted regions of the OR (Fig. 4b). A test for equal distribution of identified non-synonymous substitutions in the fifteen regions (N terminal region, the seven transmembrane regions, the three intracellular loops, the three extracellular loops, and the C terminal region) was rejected ($X^2 = 24.34$, $P = 0.042$). There was a higher than expected proportion of amino acid substitutions in the third and sixth transmembrane regions (3 TMD $P = 0.02$, 6 TMD $P = 0.005$). However, the contingency table included a number of empty cells violating an assumption of the $X^2$ test. Therefore, the non-parametric Fisher’s exact test was also conducted using 1,000,000 simulations to estimate a p-value ($P = 0.033$), which was also significant.

![Fig. 4 A Maximum likelihood tree of OR7 orthologous in *C. obliquana* (CoblOR7), *C. herana* (CherOR7), *Planotortrix octo* (PoctOR7) and *P. excessana* (PexcOR7). dN, dS and dN/dS values were generated with the M3 model](image)

### Fig. 4 A

Maximum likelihood tree of OR7 orthologous in *C. obliquana* (CoblOR7), *C. herana* (CherOR7), *Planotortrix octo* (PoctOR7) and *P. excessana* (PexcOR7). dN, dS and dN/dS values were generated with the M3 model. **B** Predicted transmembrane topology of OR7 with variable sites highlighted. Black dots resemble amino-acid substitutions in *Ctenopseustis obliquana*, whereas green dots indicate amino-acid substitutions in *C. herana* compared to a predicted ancestor. The double line indicates the membrane region, with extracellular and cytoplasmic sides labelled.
Discussion

Here we identify 47 odorant receptors expressed in adult antennae of each of the two closely-related New Zealand endemic leafroller moths, *Ctenopseustis obliquana* and *C. herana* using a transcriptome approach. Considering the number of OR genes found in other species, including 43 in *Cydia pomponella*, 21 in *Heliothis virescens*, 47 in *Manduca sexta*, 49 in *Bombyx mori* and 70 in *Epiphyas postvittana* (Krieger et al. 2002; Anderson et al. 2009; Bengtsson et al. 2012; Grosse-Wilde et al. 2011; Corcoran 2014), the number of ORs identified in the two *Ctenopseustis* species could be regarded as representative of the majority of the receptors from each of these species. Additionally to the 47 ORs identified and expressed in the antennae seven orthologous ORs of *E. postvittana* were found in the genome but could not be detected in the transcripts of *C. obliquana* and *C. herana* (EposOR8, EposOR13, EposOR17, EposOR21, EposOR23, EposOR40 and EposOR55). Unfortunately no information regarding the number of glomeruli in these species is available. In addition to mining OR genes, the transcriptomes described here will provide a useful resource for isolating and comparing other genes involved in chemosensory perception in these species, including odorant binding proteins and odour degrading enzymes.

Outside the pheromone receptor clade all OR genes described in *Ctenopseustis* are orthologous to each other and an *E. postvittana* OR. These include orthologues of Orco and the citral receptor EposOR3 that have been described previously (Jordan et al. 2009; Carraher et al. 2012). However, within the pheromone receptor clade this level of orthology breaks down with orthologues identified between *C. obliquana* and *C. herana*, but typically no counterpart in *E. postvittana*. The male biased ORs, CoblOR1 and CoblOR7 in *C. obliquana*, as well as CherOR1a, CherOR1b and CherOR7 in *C. herana*, are found within this clade where pheromone receptors from several lepidopteran species also reside (Sakurai et al. 2004; Nakagawa et al. 2005; Grosse-Wilde et al. 2007; Wang et al. 2011). CoblOR7 and CherOR7 were both found to bind the pheromone component Z8-14:OAc, whereas neither of the other male biased receptors in the pheromone receptor clade responded to any of the pheromone components presented in the HEK293 cell system. It would be interesting to test the receptors in other systems like *Xenopus oocytes* (Mitsuno et al. 2008; Nakagawa et al. 2005; Sakurai et al. 2004; Miura et al. 2010; Wang et al. 2010; Wanner et al. 2010) to verify our results. Further the presence of pheromone-binding proteins could have an effect on the ligand specificity of ORs (Grosse-Wilde et al. 2006). Although not strictly orthologous, EposOR7 was not found to bind any of *E. postvittana*’s pheromone components or any other compounds tested (Corcoran 2014). Similar to EposOR7 no direct orthologous OR could be found for EposOR1 in either of the two *Ctenopseustis* species. This OR in *E. postvittana* binds a range of terpenoids and benzoates responding best to methyl salicylate as a ligand (Jordan et al. 2009) and shows a higher rate of molecular evolution than ORs outside the sex pheromone receptor clade (Carraher et al. 2012). More recently EposOR1 has
been shown to be capable of responding to the minor pheromone component for *E. postvittana*, 
\((E,E)9,11\)-tetradecenyl acetate (Corcoran 2014). The non sex-biased ORs CoblOR6 and CherOR6, 
 together with CherOR45 and CoblOR45a and CoblOR45b are the most closely related ORs to 
EposOR1. It would be interesting to understand whether these receptors have a similar function to 
EposOR1, but unfortunately to date efforts in amplify full length versions of the genes encoding 
CoblOR6, CherOR6, CherOR45 and both versions of CoblOR45 have been unsuccessful. The 
receptors binding Z5-14:OAc in *C. obliquana* or *C. herana* have not yet been identified. The 
remaining receptors that fall into the sex pheromone receptor clade are possible candidates for being 
able to respond to this compound, even if these receptors could not be detected as male biased in RNA 
seq-count or qPCR. Similarly, receptors from *E. postvittana* that reside in this clade, but do not show 
male biased expression, have also been shown to respond to pheromone components (Corcoran 2014). 
OR30 shows higher levels of expression in male compared with female antennae, but falls outside the 
pheromone receptor clade. In the related species *E. postvittana* EposOR30 and EposOR34, like 
CoblOR30 and CherOR30, also display male biased expression and don’t seem to respond to 
pheromone components (Corcoran 2014). Further efforts are required to identify ligands for these 
male biased receptors that reside outside the pheromone receptor clade, and their orthologues. Apart 
from OR30 no orthologues of other male biased receptors in *E. postvittana* (EposOR6, EposOR07, 
EposOR34) could be found in either of the two *Ctenopseustis* species.

Of the OR genes that tend towards being female biased in their expression, OR25, OR49, OR58 and 
OR63 show similar expression differences in both *Ctenopseustis* species, suggestive of a similar role 
in both species. Notwithstanding this, we should mention that none of these genes remained female 
based in our qPCR analysis. While the female biased ORs in *E. postvittana* are largely located in a 
distinct clade separate from the pheromone receptor clade (EposOR31, EposOR36 and EposOR40), in 
*C. obliquana* and *C. herana* they dispersed throughout the phylogeny. No ligands have been identified 
for any of the female biased ORs in *E. postvittana* and none are closely related to other female biased 
receptors from *B. mori*, which respond to linalool and benzoic acid (Anderson et al. 2009). Further 
research is required to identify the ligands for these female-biased receptors in *Ctenopseustis* and 
*Epiphyas* species, which may be tuned to volatile compounds produced by host plants or male 
produced pheromones. If these receptors are involved in host finding then with the presence of both 
specialists and generalists this genera, this system may be useful in understanding the evolution of 
host specificity (Dugdale 1990). Furthermore, it has been shown in other species that males can also 
produce pheromones that are used in proximity to the female and are an important factor during 
genus, *Planotortrix*, have been observed to present hair-pencil-like structures at the tip of the 
abdomen to the female during courtship (BS, unpublished data). Such structures typically produce 
male pheromones in moths.
Electrophysiological investigations of male antennae of *C. obliquana* and *C. herana* have revealed that both species can perceive the sex pheromone components, Z5-14:OAc and Z8-14:OAc, even though Z8-14:OAc is not required to attract *C. herana* males behaviourally (Hansson et al. 1989). However, the neurophysiological response is different in each species, with *C. obliquana* possessing a neuron with a large spike amplitude that responds strongly to the major component Z8-14:OAc and another neuron with a small spike amplitude responding weakly to the minor component Z5-14:OAc. Neurons in *C. herana* showed an opposite response to these compounds (Hansson et al. 1989). As such identifying a receptor in *C. herana*, as well as *C. obliquana*, that responds to Z8-14:OAc (namely OR7) is consistent with these electrophysiological results. Presumably once the receptor responding to Z5-14:OAc is identified it too will be found to be present and expressed in the antennae of males of both species. Therefore the genetic differences explaining the changes in either neuron size or the expression of the receptor in either the large or small neuron are likely to be encoded at a locus/loci distinct to the pheromone receptors.

Interestingly, the pharmacology of the two OR7 orthologues is different. CoblOR7 is an order of magnitude more sensitive to the *C. obliquana* sex pheromone component Z8-14:OAc compared with CherOR7. Furthermore, of the compounds tested, CoblOR7 only responds to Z8-14:OAc, while in addition to this compound, CherOR7 also responds to Z7-14:OAc. This compound is not a sex pheromone component used by either of these two species, but is used as a pheromone component in the ancestral species of the genus, *C. servana* (Foster and Dugdale 1988) and species within the sister genus *Planortortrix* (Foster et al. 1986; Foster and Dugdale 1988). Considering that *C. herana* uses Z5-14:OAc solely as it’s sex pheromone, it would suggest that CherOR7 is under relaxed constraint compared with its orthologue CoblOR7. That is, mutations in CherOR7 that alter sensitivity and selectivity in this receptor are less likely to have any impact on the ability of males to locate female *C. herana*. In the closely related species of corn borer, the European and the Asian corn borer, a single mutation in the sequence of an OR gene changes the specificity of that receptor to the compound it is binding. This mutation is found in the third trans-membrane-domain (3TM) in position 148 where a threonine for alanine substitution is responsible for an alteration of sensitivity to the pheromone component E11-14:OAc (Leary et al. 2012). Interestingly, in CoblOR7 there are also amino acid differences to CherOR7 in 3TM in position 147 (alanine to glycine), 148 (serine to leucine) and 149 (phenylalanine to cysteine). Another highly variable region is located in 6TM where five amino-acid differences in position 322, 323, 324, 327 and 328 could be found. These amino-acid differences in 3TM and 6TM could be responsible for the changes in specificity and selectivity of CherOR7.

The pheromone component Z8-14:OAc is produced in the pheromone gland by a Δ10-desaturase (*desat5*) from palmitic acid and followed by a round of β-oxidation before reduction and acetylation. The basal species within the genus *Ctenopseustis, C. servana*, does not use pheromone components
that contain double bonds in an even position. This would mean that in the evolution of the genus, presumably there must have been a gain of expression of desat5 in their pheromone glands after the split from C. servana (Newcomb and Gleeson 1998; Albre et al. 2012). C. servana uses a blend containing Z5-14:OAc and Z7-14:OAc and it has been discussed that in the evolution of pheromone production, C. obliquana gained the use of Z8-14:OAc as a pheromone component (Albre et al. 2012). It has been hypothesised that South Island populations of a C. obliquana ancestor lost the expression of desat5 in the pheromone gland that gave rise to C. herana (Albre et al. 2012). Our results suggest a different model, the high substitution rate in the lineage leading to the Z8-14:OAc receptor, CoblOR7, and low substitution rate in the unspecific Z7/Z8-14:OAc orthologue, CherOR7, could suggest that C. obliquana and C. herana ancestors could have split from C. servana creating a scenario where positive selection maintained mutations that increased the specificity and sensitivity towards Z8-14:OAc in C. obliquana ancestors and a lack of selection in C. herana slowly decreased the specificity of OR7 to Z7-14:OAc. Positive selection has previously been found to act on odorant receptor orthologues in Drosophila (Tunstall et al. 2007). It has been suggested that positive selection may act on some Drosophila ORs, especially on amino acid sites, what could be responsible for altering the sensitivity of receptors towards odorants (Tunstall et al. 2007).

Changes in the sensitivity and selectivity of pheromone receptors during periods of relaxed constraint might allow a currently ‘unused’ receptor to evolve to be able to perceive a future novel component produced by a variant female. Such changes may contribute to the formation of a new species, perhaps by producing ‘rare males’ that perceive and therefore are able to ‘track’ divergent female pheromones as suggested in the “asymmetric tracking hypothesis” (Phelan 1992). In Ostrinia nubilalis it has been shown that a few ‘rare males’ are capable of responding to the pheromone blend of O. furnacalis (Domingue et al. 2007). Differences in the neurophysiological response were responsible for these rare males flying upwind to the pheromone blend of the closely related species, revealing that males can perceive a larger range of compounds, but only a few of these trigger a behavioural response depending on how the male’s olfactory receptor neurons are tuned (Domingue et al. 2007).

In C. obliquana and C. herana it also has been shown that males in both species can perceive the compound Z8-14:OAc, even though it is only used in C. obliquana as a pheromone component, but the neurological response is different (Hansson et al. 1989). Ostrinia scapulalis males possess pheromone receptors that are very specific and some that are broadly tuned also to pheromones of congeners (Miura et al. 2010). A more broadly tuned receptor as found in C. herana could provide the ability to produce males with a preference for more pheromone components when such broadly tuned receptor genes are expressed in large spike amplitude neurons or perhaps to be able to identify females moths of closely related species and respond negatively towards them. In the wasp Nasonia it has been shown that pheromone components can exist in a population without being initially perceived by the responder and a preference for this component can evolve to an additional
pheromone component leading to the evolution of a new species (Niehuis et al. 2013). Finally, a further possibility is that the receptor remains unused and becomes a pseudogene, losing its ability to produce a functional receptor as has been found in receptor genes in Drosophila (Guo and Kim 2007).
Acknowledgements

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References


Corcoran JA (2014) The identification of pheromone receptors from the lightbrown apple moth, Epiphyas postvittana. The University of Auckland, New Zealand


### Supplementary Material

#### Supplementary Material 1: Summary of metrics for the *C. herana* and *C. obliquana* transcriptome assemblies

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#### Supplementary Material 2: Primers for qPCR

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<td>------</td>
<td>--------------------------</td>
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</tr>
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<td>OR46</td>
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<td>TCGGAGCCGAGAAGCAGTCC</td>
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<tr>
<td>OR47</td>
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<td>OR52</td>
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**Supplementary Material 3: Full-length primers for cloning into pCR8/GW/TOPO vectors**

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<th>Sequence 5’-3’</th>
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**Supplementary Material 4: Full-length primers with restriction enzyme site for cloning into pcDNA 5/TO expression vector**

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<td>BamH1 RE</td>
</tr>
<tr>
<td>OR30_Cten_Apa1_R</td>
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<td>Apa1 RE</td>
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**Supplementary Material 5: Summary of read data for transcriptome assemblies**

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<td>Female antennae</td>
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<td></td>
<td>Male antennae</td>
<td>101</td>
<td>68728147</td>
<td>7675640*</td>
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Supplementary Material 7: RNA seq-count data (Fragments Per Kilobase of exon per Million fragments mapped) of all identified OR genes in *C. obliquana*.
Supplementary Material 8: RNA seq-count data (Fragments Per Kilobase of exon per Million fragments mapped) of all identified OR genes in *C. herana* male and female antennae.
Supplementary Material 9: qPCR results for investigated *C. obliquana* ORs. Mean (±SEM) relative expression to the housekeeping genes α-tubulin, β-actin and elongation factor 1α (*n* = 3) BLD = below limits of detection
Supplementary Material 10: pPCR results for investigated *C. herana* ORs. Mean (±SEM) relative expression to the housekeeping genes α-tubulin, β-actin and elongation factor 1α (n = 3) BLD = below limits of detection
Odorant receptors of the New Zealand endemic leafroller moth species Planotortrix octo and P. excessana

Bernd Steinwender123, Amali H. Thrimawithana1, Ross Crowhurst1, Richard D. Newcomb123

1 The New Zealand Institute for Plant & Food Research Limited, Auckland, New Zealand
2 School of Biological Sciences, University of Auckland, Auckland, New Zealand
3 Allan Wilson Centre for Molecular Ecology and Evolution

Keywords:
pheromone and odorant receptors, speciation, Planotortrix, leafroller

Abstract

Moths use their sense of smell to find food sources, mating partners and oviposition sites. For this they possess a family of odorant receptors (ORs). Some odorant receptors are used by both sexes whereas others are important primarily in one of the sexes. For example male moths possess ORs specifically tuned to sex pheromones produced by females. Here we identify a set of ORs from the New Zealand endemic leafroller moths Planotortrix octo (48 ORs) and P. excessana (47 ORs) using RNA Seq. Two ORs show male biased expression in the adult antennae of both species (OR7 and OR30) and one in each species was female biased (PoctOR25, PexcOR14) by qPCR. PAML analysis conducted on orthologous ORs of species from sibling genera, P. octo, P. excessana, Ctenopseustis obliquana and C. herana indicated positive selection acting on the male biased OR7 and another OR, OR64. The fact that OR7 is likely under positive selection, that it is male biased in its expression and that its orthologue in C. obliquana, CoblOR7, responded to sex pheromone components is suggestive of this receptor being important in sex pheromone reception in Planotortrix species also.
Introduction

In the insect world chemical cues play an important role in orientation and communication. Moths, for example, use their sense of smell to locate food, oviposition sites and mates (Ache and Young 2005; Stadelbacher et al. 1983). For this they use a wide range of odorant receptors (ORs) located in sensilla that are found mainly on the animal’s antennae. Male moths use specialized ORs to locate females of their own species by tracking sex pheromones produced by specialised glands in the terminal segment of the abdomen (Bradbury and Vehrencamp 1998). Females typically emit even numbered carbon chain fatty-acid derivates of 10-18 carbons in length, containing one or two double bonds along the fatty acid backbone (Linn and Roelofs 1995). For males it is important to be able to discriminate between compounds produced by con-specific or hetero-specific females because mating mistakes can be fatal for both individuals (Stadelbacher et al. 1983). Therefore little variation in pheromone reception is expected within populations.

New mating systems can only evolve if both female sex pheromone production and male reception change. In females, changes in enzymes such as desaturases or fatty acid reductases can lead to the production of novel pheromone blends (Shirangi et al. 2009; Greenberg et al. 2003; Sakai et al. 2009; Albre et al. 2012; Xue et al. 2007). Even though we are beginning to understand the female components in a sender-receiver system and how they change, the evolution of the receiver has been less intensively investigated. However to understand the evolution of new mating systems we also need to consider sex pheromone perception and what changes underpin the evolution of new mating systems and speciation (Symonds and Elgar 2008). One hypothesis in this regard is the “asymmetric tracking hypothesis”. It suggests that if females are the limiting factor within a population. Sender and response are less likely to be genetically linked providing the opportunity for pheromone perception by the male to be more variable, with some males likely to be able to detect females that produce novel pheromone blends (Phelan 1992). Indeed in some species such rare males have been found (Domingue et al. 2007). Alterations in a multigene family of receptors and differences in the structure within this family seem possible as encoding the changes in male preference (Gould et al. 2010; Wanner et al. 2010; Miura et al. 2010; Leary et al. 2012). Insect ORs are ligand-gated ion channels consisting of seven transmembrane regions with an external C-terminus and an internal N-terminus (Smart et al. 2008; Benton et al. 2006; Lundin et al. 2007). The highly conserved odorant receptor co-receptor (Orco) (Guo and Kim 2007), together with a ligand-binding OR, forms the active receptor complex. Ligand-binding ORs are more variable, especially in the N-terminal region, whereas the C-terminus is more conserved in its amino acid sequence (Tunstall et al. 2007).

ORs that bind sex pheromone components are located in specialized sensilla on the antennae called sensilla trichodea (Rumbo 1981, 1983). These long hair-like sensilla are more abundant in males than
in females in *Planotortrix* (Chapter 2). This is usually also the case for other species (Heinbockel and Kaissling 1996; Jordan et al. 2008; Mitsuno et al. 2008; Sakurai et al. 2004; Krieger et al. 2004; Koh et al. 1995) and therefore receptors expressed in these sensilla typically show higher expression in male than in female antennae. These receptors also cluster phylogenetically in the so called sex pheromone receptor clade. However in the light-brown apple moth, *Epiphyas postvittana*, and the New Zealand brown-headed leafroller moths, *Ctenopeustis obliquana* and *C. herana*, additional male biased ORs were identified that do not fall into the sex pheromone receptor clade although as yet there is no evidence that they are capable of responding to sex pheromone components (Chapter 3; Corcoran 2014).

Species of the endemic New Zealand brown-headed leafroller, *Ctenopeustis* (5 species), and green-headed leafroller, *Planotortrix* (7 species), are found throughout New Zealand with some occurring all over the country and others restricted to certain regions (Dugdale 1990; Newcomb et al. 2014 in press) and preferences for host-plants ranging from specialists to highly polyphagous (Crosby et al. 1976). The most recent common ancestor for the two sister genera *Ctenopeusis* and *Planotortrix* is thought to have existed about 5 million years ago. Some species within the genus *Ctenopeustis* have an estimated most recent common ancestor of less than 500 000 years ago with some sibling species within *Planotortrix* derived even more recently (Langhoff et al. 2009). Together with the Australian light-brown apple moth, *Epiphyas postvittana*, the polyphagous species *C. obliquana*, *C. herana*, *P. octo* and *P. excessana* form a complex of pest species in New Zealand (Wearing et al. 1991) that have been investigated in some detail (Suckling and Brockerhoff 2010; Newcomb et al. 2014 in press).

Females of *Ctenopeustis* and *Planotortrix* species produce blends consisting of unsaturated tetradecenyl acetates to attract con-specific males (Roelofs and Brown 1982). Typically double bonds are found at the 5, 7, 8 or 9 positions of the fatty acid-backbone of the molecule (Roelofs and Brown 1982; Newcomb and Gleeson 1998; Foster et al. 1986). While the compounds differ in the position of the double bond in the fatty-acid backbone, they are always in the Z-isomer configuration (Newcomb and Gleeson 1998; Foster et al. 1986). Females in the four polyphagous species use different pheromone blends to attract conspecific males: *C. obliquana* uses (Z)-5-tetradecenyl acetate (Z5-14:OAc) and (Z)-8-tetradecenyl acetate (Z8-14:OAc) in a ratio of 20:80, *C. herana* uses solely Z5-14:OAc, *P. octo* uses Z8-14:OAc as a pheromone only and *P. excessana* females produce a blend of Z5-14:OAc and (Z)-7-tetradecenyl acetate (Z7-14:OAc) in a ratio of 60:40 (Dugdale 1990). The expression of different desaturase genes has been shown to be responsible for to producing the different pheromone components (Albre et al. 2012; Albre et al. 2013). The Δ10-desaturase gene in *Planotortrix* is thought to be differentially regulated by a trans-acting repressor and a cis-regulatory mutation in an activator binding site within the desaturase’s promoter region (Albre et al. 2013).
As well as research conducted on female sex pheromone production in New Zealand endemic leafroller moths, sex pheromone reception has also been investigated. A study focussing on male sex pheromone perception in the genus *Ctenopseustis* showed in electrophysiological experiments that males of *C. herana* can detect the component Z8-14:OAc despite it not being a pheromone component in this species, but used by the sibling species *C. obliquana* with a substantially different neurological response to the component (Hansson et al. 1989). Males of *C. obliquana* possess a large spike amplitude cell that responds strongly to Z8-14:OAc, whereas *C. herana* males have a small spike amplitude cell that responds weakly to this component. Functional analysis in HEK293 cells of male biased receptors showed that OR7 in *C. obliquana* (CoblOR07) responds only to Z8-14:OAc, but its orthologue in *C. herana* (CherOR07) responds to Z7-14:OAc in addition to Z8-14:OAc. OR7 clusters phylogenetically in the sex pheromone receptor clade and investigations of both orthologs indicate that CoblOR7 is under positive selection, presumably because it is important in *C. obliquana* to have a receptor that is highly specific to the sex pheromone component Z8-14:OAc. Considering that *C. herana* uses Z5-14:OAc alone as its sex pheromone, OR7 in *C. herana* seems to be under relaxed constraint (Chapter 2). Furthermore the identification of ORs in *C. obliquana* and *C. herana* using transcriptome databases from male and female antennae as well as quantitative real-time PCR (qPCR) revealed high similarity of orthologuous OR genes and male biased expression of the same two genes in both sibling species, OR07 and OR30.

Here we apply transcriptomic and bioinformatic tools to identify odorant receptors in the New Zealand green-headed leafroller species, *P. octo* and *P. excessana*. We identify candidate sex pheromone receptors through examining expression differences of OR genes between the male and female antennae and conduct dN/dS analysis to test for evidence of selection acting on these genes.
Material and Methods

**Insects**

All insects were reared in the Plant & Food Research insect rearing facility at the Mt Albert Research Centre, Auckland, New Zealand. The *P. octo* colony originated from collections made in Canterbury in 1982 and the *P. excessana* culture was established from moths collected at Dunedin in 1998. Eggs were collected and kept in a humid environment. Larvae were reared individually in small glass tubes and fed on a general purpose diet (Clare and Singh 1988) at 18°C. Pupae and adults were kept on a 16:8 light cycle at 20°C. Moths were provided with water soaked in cotton cloth.

**Nucleic acid isolation**

RNA isolation for transcriptome sequencing was executed from batches of 100 male and female antennae each dissected from 2-3 days old adult moths. RNA was extracted and purified using TRIzol (Invitrogen, Carlsbad, CA, USA) and total RNA was DNase treated using the TURBO DNA-free Kit (Life technologies, Carlsbad, CA, USA) as described in Chapter 3.

RNA for quantitative RT-PCR (qPCR) experiments was also made from antennae dissected from 2-3 days old adults, as well as whole bodies, using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. The initial screening for expression differences of ORs in male and female antennae was conducted with ten pairs of antennae with subsequent qPCR experiments from RNA of single antennae pairs. RNA was treated with DNase (DNaseI amplification grade, Invitrogen) following the manufacturer’s instructions and cDNA was synthesised using iScript cDNA Synthesis Kit (Bio-Rad) as described in Chapter 3.

**Next-generation sequencing and bioinformatics**

RNAseq libraries were constructed from both male and female adult antennae of *P. octo* and *P. excessana* using Illumina’s standard protocols and sequenced at Macrogen (Seoul, South Korea). Quality score analysis on the read pairs for each library was undertaken using FastQC (FastQC 2008).

In-house Perl scripts were used to trim all reads by 13 bases at their 5’ ends and remove any read pairs containing Ns and mononucleotides. Mitochondrial contamination was removed by mapping RNASeq read pairs to a reference mitochondrial genome of *P. octo* assembled from a draft genome. Mapping was performed using bowtie (version 1.0.0) (Langmead et al. 2009) with the reads mapping to the mitochondrial genome being removed. Read pairs were trimmed to a minimum quality threshold of 20 using fastq-mcf from the ea-utils package (Aronesty 2011). Duplicates within the read files for *P. octo* were removed using in-house Perl scripts prior to assembly, while the redundancy in the *P. excessana* sequences was removed after the assembly using cd-hit (Li and Godzik 2006). De novo assembly of the processed reads was performed for each of the individual libraries (Supplementary Material 1) with trans-ABySS (version 1.3.2) (Robertson et al. 2010), where a k-mer series of 31 to 75 with an
increment of two bases was used for the libraries of *P. octo*, whereas k-mer values from 31 to 71 were used for the *P. excessana* libraries.

To identify candidate ORs, tblastn (Altschul et al. 1990) was used with amino-acid sequences of ORs from the light-brown apple moth *Epiphyas postvittana* (Corcoran 2014) as well as the ORs from each of the brown-headed leafroller moth species *Ctenopseustis obliquana* and *C. herana* (Chapter 3) as queries. Full length sequences of ORs were obtained using tblastn against the transcriptomes of male and female antennae from *P. octo* and *P. excessana*. In some cases a draft genome of *P. octo* (unpublished data) was used to extend sequences. The processed RNASeq reads were mapped onto a constructed set of OR sequences using bowtie (version 2.1.0) (Langmead and Salzberg 2012). The resulting alignment was then used to obtain expected read counts using multiBamCov from the bedtools package (v2.16.2) (Quinlan and Hall 2010) and cufflinks (v2.1.1) (Trapnell et al. 2010) for Fragments Per Kilobase of transcript per Million reads (FPKM).

Acquired sequence data was edited and aligned in Geneious (Kearse et al. 2012) using ClustalW with *C. obliquana, C. herana* and *E. postvittana* sequences. The model for phylogenetic trees was chosen by ModelTest (Posada and Crandall 1998) and maximum likelihood trees were generated in Mega (Hall 2013). The dN and dS rates were estimated using codon-based substitution models in PAML version 4.7 (Yang and Nielsen 2000) by using M0 as the model with one fixed ω ratio and the model M3, which has three categories of site with a free ω ratio for each site class (Yang and Nielsen 2000). Transmembrane domains were predicted using SPLIT 4.0 (Juretic et al. 2002) at the transmembrane prediction server (http://split4.pmfst.hr/split/4/). The topology diagram was constructed using TOPO2 Transmembrane Protein Display (Johns 2005) by the server at UCSF (http://www.sacs.ucsf.edu/TOPO2).

**Quantitative Real-Time PCR**

Expression levels of ORs in male and female antennae and bodies were determined relative to α-tubulin, β-actin and elongation factor 1α (Turner et al. 2006). Primers were designed to receptors found in *P. octo* and tested on the orthologous receptors from the sibling species *P. excessana* (Supplementary Material 2). Experiments were executed as in Chapter 3 using 20 ng of cDNA in a final reaction volume of 10 µl over 45 cycles using a LightCycler480 Real-Time instrument (Roche Diagnostics, Basel, Switzerland). The data analysis was also conducted as described in Chapter 3, with the determination of the amplification efficiency and the cycle threshold values for each reaction conducted using the software LinRegPCRv11 (Ramakers et al. 2003) and expression levels were calculated as described in Corcoran 2014 using a modified version of the ΔCp method (Pfaffl 2001; Livak and Schmittgen 2001; Albre et al. 2013).
Results

Antennal transcriptomics and the OR genes

In total four RNAseq libraries were generated from the antennae of 2-3 day old adult male and female *P. octo* and *P. excessana* generating from 56,971,439 to 61,132,402 raw sequence reads (Supplementary Material 1). Assemblies from RNAseq data ranged in the number of contigs from 125,512 to 228,676 (Supplementary Material 3). Forty-eight OR genes from *P. octo* and 47 from *P. excessana* were identified and corresponding protein sequences were derived. Except for PexcOR16, all ORs outside the sex pheromone receptor clade were represented by orthologous sets in both *Planotortrix* species (Figure 1A). A comparison with ORs identified in species of the sister genus *Ctenopseustis, C. obliquana* and *C. herana*, (Chapter 3) revealed that OR60 is exclusively expressed in antennae of *P. octo* and *P. excessana* and OR11 is found in *C. obliquana* and *C. herana* antennae only. Orthologous ORs within *Planotortrix* share levels of amino acid identity between 87.7% and 100% (Supplementary Material 4). The clade containing sex pheromone receptors from other moth species including *C. obliquana* and *C. herana* is supported by bootstrap analysis (81% from 1000 bootstrap replicates). Within the sex pheromone receptor clade all identified ORs are present as orthologs in all four New Zealand endemic leafroller species apart from OR6 that was not found among the *P. excessana* transcripts (Figure 1B).

Gene expression of ORs

Sex pheromone receptors are in general more highly expressed in male antennae than in female antennae (Krieger et al. 2004; Grosse-Wilde et al. 2010), therefore we identified receptors with male biased expression in adult antennae (Supplementary Material 5 and 6). Using a two-fold cut off in FPKM scores two OR genes in each of *P. octo* and *P. excessana* were identified as male biased, OR7 and OR30. Orthologs of these ORs are also found to be male biased in species of the sister genus *Ctenopseustis* with OR7 falling into the sex pheromone receptor clade and OR30 not falling into this clade (Figure 1C). Through RNAseq counting with a minimum count of 10,000 FPKM and a two-fold cut off also revealed ten female biased ORs in *P. octo* (PoctOR10, PoctOR14, PoctOR22, PoctOR25, PoctOR27, PoctOR39, PoctOR47, PoctOR64, PoctOR66 and PoctOR71) and nine in *P. excessana* (PexcOR14, PexcOR22, PexcOR25, PexcOR26, PexcOR39, PexcOR57, PexcOR64 and PexcOR66.)
Figure 1. Phylogenetic analyses of odorant receptors from *Planotortrix octo*, *P. excessana*, *Ctenopseustis obliquana* and *C. herana*. A. Circle tree of odorant receptors from *Planotortrix octo*, *P. excessana*, *Ctenopseustis obliquana* and *C. herana* together with *Epiphyas postvittana*. The tree is rooted with the odorant receptor co-receptor Orco. The positions of OR07 and OR30 are indicated with arrows and the sex pheromone receptor clade is highlighted with a semi-circle (Letunic and Bork 2007, 2011). B. Phylogeny of the sex pheromone receptor clade containing odorant receptor genes of *P. octo*, *P. excessana*, *C. obliquana*, *C. herana* and *E. postvittana*. The odorant receptor, OR7, that shows male biased expression in all four New Zealand endemic leafroller species is highlighted in blue. C. Phylogeny of odorant receptors from species with male biased expression from outside the pheromone receptor clade. The odorant receptor, OR30, with higher gene expression in male compared with female adult antennae in all five species is highlighted in blue.
Twenty-five OR genes in *P. octo* and twenty-six in *P. excessana* were investigated for their levels of gene expression using qPCR (Supplementary Material 7 and 8). Among the investigated genes was the male biased receptors from the FPKM analysis, OR7 from the pheromone receptor clade, as well as OR30. Both genes were also male biased by qPCR in both species (Figure 2 A and B; PocTO7 \(P = 0.014\); PexcOR7 \(P = 0.009\); PocTOR30 \(P = 0.014\); PexcOR30 \(P = 0.004\)). In addition to the male biased receptors, female biased receptors were also identified. In *P. octo* one receptor was confirmed as being more highly expressed in female than male antennae (PocTO25, \(P = 0.002\)), but not PocTO10 (\(P = 0.142\)), PocTO14 (\(P = 0.246\)), PocTOR22 (\(P = 0.859\)), PocTOR27 (\(P = 0.743\)), PocTOR39 (\(P = 0.382\)), PocTOR47 (\(P = 0.657\)) and one OR from *P. excessana* (PexcOR14 \(P = 0.020\)) but not PexcOR22 (\(P = 0.552\)), PexcOR25 (\(P = 0.564\)), PexcOR26 (\(P = 0.473\)), PexcOR32 (\(P = 0.211\)) and PexcOR39 (\(P = 0.126\)). No expression differences in any of the other ORs that were investigated were observed.

**Figure 2.** Relative expression of the odorant receptors OR7 and OR30 in male (blue) and female (red) antennae of adult **A. Planotortrix octo** and **B. P. excessana.** CT values normalised to the housekeeping genes α-tubulin, β-actin and elongation factor 1α. BLD = below limits of detection.

**Sequence comparison of ORs**

Full length coding sequences for 36 OR could be derived for receptors from *C. obliquana, C. herana, P. octo* and *P. excessana*. These sequences were tested for positive selection by comparing the M0 and M3 models for \(\omega\) (Table 1). The likelihood ratio test revealed positive selection in two ORs. The male biased OR7 and OR64, which is female biased in *C. herana, P. octo* and *P. excessana*. OR7 is male biased in all four sibling species. The results from the test for selection of the orthologues CoblOR7 in *C. obliquana* and CherOR7 in *C. herana* have been stated and discussed in Chapter 3. A likelihood ratio test executed to find evidence for positive selection by comparison M0 vs M3 in *Planotortrix* was significant (\(P = 0.003; 2\Delta l = 17.77\)). A comparison of OR7 orthologs in *Planotortrix* showed a dN/dS ratio of \(\omega = 0.8426\) for *P. octo* (43.8 non-synonymous and 18.8 synonymous changes) and \(\omega = 0.0709\) for *P. excessana* (2.2 non-synonymous and 11.4 synonymous changes).
(Figure 3A). There are 71 nucleotide differences between PoctOR7 and PexcOR7 comprising 26 synonymous and 45 non-synonymous substitutions (Figure 3B). A test for equal distribution of non-synonymous substitutions in the fifteen regions (N terminal region, seven transmembrane regions, three intracellular loops, three extracellular loops, and the C terminal region) was not rejected ($X^2 = 14.84, P = 0.39$).

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Table 1. Likelihood ratio tests between models M0 and M3 (Mfree model) showing log likelihood for each model and twice the difference of these log likelihoods between the two models ($X^2$). Degrees of freedom is equal to the number of branches of the tree minus one, 5 in all cases.
Figure 3. PAML analysis of OR7 across endemic New Zealand leafroller moths. A. Maximum likelihood tree of OR7 orthologues from *C. obliquana* (CoblOR7), *C. herana* (CherOR7), *P. octo* (PoctOR7) and *P. excessana* (PexcOR7). $d_N$, $d_S$ and $d_N/d_S$ values were generated using the M3 model. B. Predicted transmembrane topology of OR7 with variable sites highlighted. Red dots indicate the position of amino-acid substitutions in *Planotortrix octo*, and black dots amino-acid substitutions in *P. excessana*. The double line indicates the transmembrane region, with extracellular and cytoplasmic sides labelled.

The other receptor that was male biased in *P. octo* and *P. excessana* is OR30. The likelihood ratio comparing M0 vs M3 was not significant ($P = 0.24$; $2\Delta l = 8.0071$). The $d_N/d_S$ ratio for CoblOR30 in *C. obliquana* was 0.5311 (8.0 non-synonymous and 5.4 synonymous substitutions), in *C. herana* $\omega = 0.0476$ (1.1 non-synonymous and 8.0 synonymous substitutions), in *P. octo* $\omega = 2.5394$ (7.4 non-synonymous and 1.0 synonymous substitutions) and for *P. excessana* $\omega = 0.5854$ (3.6 non-synonymous and 2.2 synonymous substitutions) (Figure 4A). We note that because the number of substitutions in OR30 is low, small bias in the ratio of synonymous to non-synonymous changes has a large impact on $\omega$ values. There are 14 nucleotide differences between PoctOR30 and PexcOR30 which included three synonymous and 11 non-synonymous substitutions (Figure 4B). Between CoblOR30 and CherOR30 there are 21 nucleotide differences comprising 11 synonymous and 10 non-synonymous substitutions (Figure 4C). No deviations from an equal distribution of non-synonymous
substitutions among the fifteen regions of the receptor were revealed for OR30 from *P. octo* or *P. excessana*.

**Figure 4.** PAML analysis of OR30 across endemic New Zealand leafroller moths. A. Maximum likelihood tree of OR30 orthologues from *C. obliquana* (CoblOR30), *C. herana* (CherOR30), *P. octo* (PoctOR30) and *P. excessana* (PexcOR30). dN, dS and dN/dS values were generated using the M3 model. Predicted transmembrane topology of OR30 with variable sites highlighted in B. *Ctenopseustis*: Black dots indicate amino-acid substitutions in *C. obliquana*, green dots amino-acid substitutions in *C. herana*, and black-green dots indicate independent substitutions in *C. obliquana* and *C. herana* and *C. Planotortrix*: red dots indicate amino-acid substitutions in *P. octo*, black dots indicate substitutions in *P. excessana*, red-black dots resemble independent substitutions in *P. octo* and *P. excessana*. The double line indicates the transmembrane region, with extracellular and cytoplasmic sides labelled.

Odoran receptor 64 is female biased in *C. herana, P. octo* and *P. excessana*. The likelihood ratio test comparing M0 vs. M3 was significant (*P = 0.01; 2Δl = 15.00*). The dN/dS ratio for CoblOR64 in *C. obliquana* was calculated to be $\omega = 0.4649$ (30.4 non-synonymous and 22.3 synonymous substitutions), in *C. herana* $\omega = 0.1004$ (4.2 non-synonymous and 14.3 synonymous substitutions), in *P. octo* $\omega = 0.2939$ (3.1 non-synonymous and 3.6 synonymous substitutions) and in *P. excessana* $\omega = 0.0062$ (5.9 non-synonymous and 5.1 synonymous substitutions) (Figure 5A). Between PoctOR64 and PexcOR64 there are 17 nucleotide differences comprising eight synonymous substitutions and nine non-synonymous substitutions (Figure 5B). A test for equal distribution of the non-synonymous substitutions in the fifteen regions was rejected ($X^2 = 24.44$, *P = 0.04*). There was a higher than
expected proportion of amino acid substitutions in the second transmembrane region and first internal loop (2 TM $P = 0.0004$, 1 IL $P = 0.014$). However, the contingency table included a number of empty cells violating an assumption of the $X^2$ test. Therefore, the non-parametric Fisher’s exact test was also conducted using 1,000,000 simulations to estimate a p-value ($P = 0.098$), which was not significant.

Between CoblOR64 and CherOR64 there are 65 nucleotide differences comprising 31 synonymous substitutions and 34 non-synonymous substitutions (Figure 5C). A test for equal distribution of these non-synonymous substitutions in the fifteen regions was also rejected ($X^2 = 24.29$, $P = 0.04$). There was a higher than expected proportion of amino acid substitutions in the second and fourth transmembrane regions (2 TM $P = 0.02$, 4 TM $P = 0.004$). Again, the contingency table included a number of empty cells violating an assumption of the $X^2$ test. Because of this the non-parametric Fisher’s exact test was also executed using 1,000,000 simulations to estimate a p-value ($P = 0.028$), which was significant.

**Figure 5.** PAML analysis of OR64 across endemic New Zealand leafroller moths. A. Maximum likelihood tree of OR64 orthologues in *C. obliquana* (CoblOR64), *C. herana* (CherOR64), *P. octo* (PoctOR64) and *P. excessana* (PexcOR64). dN, dS and dN/dS values were generated using the M3 model. Predicted transmembrane topology of OR64 with variable sites highlighted in B. *Ctenopseustis*: Black dots indicate amino-acid substitutions in *C. obliquana*, green dots amino-acid substitutions in *C. herana*, and black-green dots indicate independent substitutions in *C. obliquana* and *C. herana* and C. *Planotortrix*: red dots indicate amino-acid substitutions in *P. octo*, black dots indicate substitutions in *P. excessana*, and red-purple dots indicate independent substitutions in *P. octo* and *P. excessana*. The double line indicates the transmembrane region, with extracellular and cytoplasmic sides labelled.
Discussion

Using a transcriptomics approach we have identified 48 odorant receptors (ORs) from *P. octo* and 47 from *P. excessana*. The number of ORs identified in the two *Planotortrix* species is similar to the number identified in species of the sister genus *Ctenopseustis* (Chapter 3), but less than the number identified in the related tortricid species *Epiphyas postvittana* were 70 ORs have been identified (Corcoran 2014). In another tortricid moth, *Cydia pomonella*, 43 OR have been identified (Bengtsson et al. 2012) and 50 glomeruli were found (Trona et al. 2010). To date no information concerning the number of glomeruli in these species is available. It remains possible that more ORs remain to be identified in these species. The transcriptomes used for the identification of ORs and isolation of their genes can be used in the future to find other genes encoding proteins involved in the process of chemoreception such as other classes of receptors (ionotropic receptors, gustatory receptors), odorant/pheromone binding proteins or odorant degrading proteins.

Within the pheromone receptor clade the presence of orthologous genes between *E. postvittana* and the two *Planotortrix* species is very low compared to ORs outside the clade. This was also observed between ORs from *E. postvittana* and species within the genus *Ctenopseustis* (Chapter 3). However, between the genus *Planotortrix* and *Ctenopseustis*, even in the highly variable pheromone receptor clade orthologuous genes were identified. This is despite the fact that ORs within the pheromone receptor clade seem to evolve faster than other ORs (Carraher et al. 2012).

All OR genes outside the pheromone receptor clade are present as highly conserved orthologous pairs in the two *Planotortrix* species except for OR16 that is only found expressed in the antennae of adult *P. excessana*. Similarly between the two genera *Planotortrix* and *Ctenopseustis* all OR genes form orthologous sets, except for OR60 that is present only in *Planotortrix* and OR11 that is present only in *Ctenopseustis*. Among these genes is the odorant receptor co-receptor (Orco) and OR3 which have been described previously (Carraher et al. 2012).

The male biased receptors PoctOR7 in *P. octo* and PexcOR7 in *P. excessana* reside within the clade where pheromone receptors of several lepidopteran species are found (Sakurai et al. 2004; Nakagawa et al. 2005; Grosse-Wilde et al. 2007; Wang et al. 2011). PoctOR7 has a higher dN/dS value than PexcOR7. Though not statistically significant, a higher proportion of substitutions were observed in the sixth transmembrane region (Figure 3B) of PoctOR7. This is also the case in the orthologous receptor CoblOR7 in *C. obliquana* where more substitutions were also found (Chapter 3). PoctOR7 and PexcOR7 have been functionally tested in HEK293 cell assays but to date efforts to find a binding ligand for these two receptors have been unsuccessful. The orthologues CoblOR7 and CherOR7 Are functional in HEK293 cells however, responding to the sex pheromone component (Z)-8-tetradecenyl acetate (Z8-14:OAc) which is used in *C. obliquana* as the major component of the sex pheromone blend. Interestingly CherOR7 in *C. herana* responded to Z8-14:OAc and (Z)-7-tetradecenyl acetate
(Z7-14:OAc) even though neither of these components is used as a pheromone in this species (Chapter 3). It has been hypothesised that CoblOR7 is under positive selection, whereas CherOR7 is under relaxed constraints giving it the opportunity to mutate without fitness consequences for C. herana males (Chapter 3). Females in P. octo use Z8-14:OAc as a pheromone and P. excessana females produce Z7-14:OAc and (Z)-5-tetradecenyl acetate (Z5-14:OAc). Following on the assumption made in Chapter 3 that OR7 is ancestrally the receptor responding to Z7-14:OAc, positive selection may have acted on amino-acid substitutions in PoctOR7 that render it more sensitive and selective for Z8-14:OAc. In contrast the very low numbers of non-synonymous substitutions in PexcOR7 compared to a predicted ancestor would indicate stabilising selection since P. excessana uses Z7-14:OAc as a pheromone. Apart from OR7 only one other OR was found to be under positive selection, OR64 (see below). The lack of selection in other ORs together with results acquired from cell assays on CoblOR7 and CherOR7 (Chapter 3), indicate an important role for OR7 as a pheromone receptor in New Zealand endemic leafroller moths.

The second receptor identified as male biased in all four New Zealand endemic leafroller species is OR30. This receptor resides outside the pheromone receptor clade and is also present as an orthologue in E. postvittana, where it is also more highly expressed in male than in female antennae (Corcoran 2014). Compared to OR7, OR30 shows fewer amino acid differences when comparing with orthologues of the sibling species C. obliquana/C. herana and P. octo/P. excessana. One reason could be the higher rate of evolution of OR7, observed for receptors within the pheromone receptor clade (Carraher et al. 2012). Another explanation could be the function of this receptor. Even though no functional data are yet available for OR30 in either of the New Zealand native leafroller or E. postvittana, this OR could be a candidate for being the receptor binding Z5-14:OAc in Ctenopseustis and Planotortrix. The compound Z5-14:OAc is used by C. obliquana, C. herana and P. excessana as a sex pheromone component and also used in other species within the genera Ctenopseustis and Planotortrix (see (Newcomb and Gleeson 1998) for a summary of pheromones-blends used in the New Zealand endemic leafroller). Therefore lower levels of sequence variation could be expected as stabilizing selection may prevent changes in amino acid sequence.

One OR in P. octo (PoctOR25) and a further in P. excessana (PexcOR14) was confirmed as being female biased in their expression in antennae by qPCR (Supplementary Material 7 and 8). Even though not significantly female biased by qPCR in either of the two Planotortrix species, OR22 showed a trend towards higher expression in female than in male antennae by RNAseq-counting. In C. obliquana, C. herana and also in E. postvittana OR22 was also detected as being female biased (Chapter 3; Corcoran 2014). In total three orthologous receptor genes were more highly expressed in female antennae than in males in all four species of New Zealand endemic leafroller moths (OR14, OR22 and OR25; Supplementary Material 5, 6 and Chapter 3), suggesting a possible important role
for these receptors in adult females. Likelihood ratio tests indicated possible positive selection in the receptor CoblOR64. Interestingly this receptor is not female biased in *C. obliquana* but shows higher levels of expression in female antennae than in male antennae in *C. herana*, *P. octo* and *P. excessana* by RNA-seq counting (Online resource 1 and 2 and Chapter 3). The results from the PAML analysis as well as the expression patterns in the four species could imply that OR64 has taken on a new role in *C. obliquana* that is equally important for males and females. Further investigation of the female biased receptors in these generalists but also in specialist species within the New Zealand native leafroller complex will be useful to understand the evolution of host specificity (Dugdale 1990). Also it has been shown in other tortricid moths that males too can produce pheromones. These pheromones are used in closed range and are an important factor during courtship (Davie et al. 2010; Hillier and Vickers 2004, 2011). Female biased ORs in *Planotortrix* and *Ctenopseustis* species could be involved in detecting pheromones produced by males and facilitate mate recognition.
Acknowledgements

We would like to thank Anne Barrington for supplying moths. Funding was provided by the Allan Wilson Centre for Molecular Ecology and Evolution.


Corcoran JA (2014) The identification of pheromone receptors from the lightbrown apple moth, Epiphyas postvittana. The University of Auckland, New Zealand


Supplementary Material

Supplementary Material 1: Summary of read data for transcriptome assemblies

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Supplementary Material 2: Primers for qPCR

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**Supplementary Material 3: Summary of metrics for *P. excessana* and *P. octo* transcriptome assemblies**

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Supplementary Material 5: RNA-seq count data (FPKM: Fragments Per Kilobase of exon per Million fragments mapped) of all identified OR genes in *P. octo*.

- **FPKM**

- Male antennae
- Female antennae
Supplementary Material 6: RNA-seq count data (Fragments Per Kilobase of exon per Million fragments mapped) of all identified OR genes in P. excessana.
Supplementary Material 7: qPCR results for investigated *P. octo* ORs. Mean (±SEM) relative expression to the housekeeping genes α-tubulin, β-actin and elongation factor 1α (n = 3)
Supplementary Material 8: pPCR results for investigated *P. excessana* ORs. Mean (±SEM) relative expression to the housekeeping genes α-tubulin, β-actin and elongation factor 1α (*n* = 3).
Final conclusion & future directions

With this PhD project I aimed to identify and characterise sex pheromone receptors (PRs) of the New Zealand native leafroller moth species *Ctenopseustis obliquana*, *C. herana*, *Planotortrix octo* and *P. excessana*, to gain more understanding of the evolution of sex pheromone reception in male moths. In the process of doing so I also identified a number of olfactory receptors (ORs), some of them showing female biased expression. Additionally the ultrastructure of the antennae in both males and females of the four species has been investigated. With the existing information about the genera *Planotortrix* and *Ctenopseustis* and the knowledge gained during my research, the New Zealand endemic leafroller moths become a more valuable model system to address further questions related to the evolution of mating systems in moths, host specificity and even pest control.

In my first manuscript I investigated the ultrastructure of male and female antennae. All four species showed the same gross morphology and possessed the same six types of sensilla: Sensilla trichodea (type I, II, III), sensilla basiconica, sensilla auricillia, sensilla chaetica, sensilla coeloconica and sensilla styloconica. Males possess an additional type of s. trichodea (type I) that is missing in females. Sensilla trichodea are involved in sex pheromone detection (Rumbo 1983, 1981; Krieger et al. 2005; den Otter et al. 1978; Hansson et al. 1989) and are male biased in other species (Jordan et al. 2008; Koh et al. 1995; Gomez and Carrasco 2008). The sensilla identified and described here are useful for further applications such as electrophysiological studies in males as well as in females.

In my second and third manuscript I identified ORs and PRs of the two sibling species in *Ctenopseustis* and *Planotortrix*. Using bioinformatic tools as well as molecular biology techniques 47 ORs in each *C. obliquana*, *C. herana* and *P. excessana* and 48 ORs in *P. octo* were identified. Considering the number of OR genes identified in other species, 43 ORs were identified in *Cydia pomponella*, 21 in *Heliothis virescens*, 47 in *Manduca sexta*, 49 in *Bombyx mori* and 70 in *Epiphyas postvittana* (Krieger et al. 2002; Anderson et al. 2009; Bengtsson et al. 2012; Grosse-Wilde et al. 2011; Corcoran 2014), the number of ORs identified here could be regarded as representative. Because only males possess sensilla trichodea type I on their antennae, PR candidates were expected to be expressed more highly in male than in female antennae. Three male biased ORs in *C. obliquana*, four in *C. herana* and two in each *P. octo* and *P. excessana* were identified. Two receptors were male biased in all four species, OR7 and OR30. Odorant receptor 7 falls phylogenetically into the sex pheromone receptor clade and OR30 does not. For functional characterisation the identified PR candidate genes were expressed in HEK293 cells. Calcium assays conducted with the most common pheromone compounds found in *Ctenopseustis* and *Planotortrix* were successful for OR7 in *C. obliquana* and *C. herana*. The results gave very interesting insight in the pharmacology of CoblOR7 and CherOR7. CoblOR7 is an order of magnitude more sensitive to the *C. obliquana* sex pheromone component (Z)-8-tetradecenyl acetate (Z8-14:OAc) compared with CherOR7. Furthermore, of the
compounds tested, CoblOR7 only responds to Z8-14:OAc, while in addition to this compound, CherOR7 also responds to (Z)-7-tetradecenyl acetate (Z7-14:OAc). While Z8-14:OAc is a pheromone component for *C. obliquana* but not *C. herana*, Z7-14:OAc is not a pheromone component used by either of these two species, but is used as a pheromone component in the ancestral species of the genus, *C. servana* (Foster and Dugdale 1988), and species within the sister genus *Planotortrix* (Foster and Dugdale 1988; Foster et al. 1986). Considering that *C. herana* uses Z5-14:OAc solely as its sex pheromone, it would suggest that CherOR7 is under relaxed constraint compared with its orthologue CoblOR7. A test for positive selection indicates that CoblOR7 is under selection while CherOR7 is not. CherOR7 being under relaxed constraint would mean that mutations in CherOR7, that alter sensitivity and selectivity in this receptor, are less likely to have any impact on the ability of males to locate female *C. herana*. In the European and the Asian corn borer, a single mutation in the sequence of an OR gene changes the specificity of that receptor to the compound it is binding. This mutation is found in the third trans-membrane-domain (3TM) (Leary et al. 2012). In CoblOR7 in the same region amino acid differences to Cher OR7 in 3TM could be identified. Another highly variable region was located in 6 TM. These differences could be responsible for changes in specificity and selectivity of Cher OR7. The high substitution rate in CoblOR7 and the low substitution rate in the unspecific CherOR7 could suggest that *C. obliquana* and *C. herana* ancestors could have split from *C. servana*, which uses Z7-14:OAc as a pheromone component, creating a scenario where positive selection maintained mutations that increased the specificity and sensitivity towards Z8-14:OAc in *C. obliquana* ancestors and a lack of selection in *C. herana* slowly decreased the specificity of OR7 to Z7-14:OAc.

In *Planotortrix*, PoctOR7 showed a higher number of amino acid substitutions compared to PexcOR7 with higher (but not significant) amino acid substitutions in TM6. *Planotortrix octo* uses Z8-14:OAc as its sole sex pheromone and *P. excessana* uses Z7-14:OAc and (Z)-5-tetradecenyl acetate (Z5-14:OAc) in its sex pheromone blend. Unfortunately efforts to functionally test PoctOR7 and PexcOR7 in HEK293 cells, has been unsuccessful. Even though it was not possible to functionally characterise PoctOR7 and PexcOR7, a similar scenario as suggested for CoblOR7 and CherOR7 could be possible. Following the assumption that OR7 is ancestrally the receptor responding to Z7-14:OAc, as suggested in *Ctenopseustis*, positive selection may have acted on amino-acid substitutions in PoctOR7 that render it more sensitive and selective for Z8-14:OAc. In contrast the very low numbers of non-synonymous substitutions in PexcOR7 compared to a predicted ancestor would indicate stabilising selection since *P. excessana* uses Z7-14:OAc as a pheromone.
Future directions

Even though the identification and characterisation of two PR candidates, namely CoblOR7 and CherOR7, was successful, their orthologuous receptors in *P. octo* (PoctOR7) and *P. excessana* (PexcOR7) still need to be functionally characterised. I would suggest repeat transfections into HEK293 cells and repeat cell assays with the unidentified receptors to verify their status as PRs.

Once PR candidate receptors in these species have been characterised experiments could be extended to all New Zealand native leafroller species. A characterisation of OR7 in all species with HEK293 cells would be a great asset to the understanding of the evolution of sex pheromone reception in the genera *Ctenopseustis* and *Planotortrix* and will most likely help us to understand the evolution of new pheromone races and speciation through the evolution of sex pheromones in insects.

Admittedly the Paml analysis executed in this thesis shows its weakness because of the low numbers of genes used. It would be important to repeat the analysis for positive selection in OR7 with all 12 species to get more powerful results.

The PR candidates CoblOR7, CherOR7, PoctOR7 and PexcOR7 can be used to execute mutagenesis experiments to identify amino acid substitutions responsible for the change in specificity of OR7 in *Ctenopseustis* and *Planotortrix*. With similar experiments it would be possible to generate an ancestral OR7. Functional characterisation of the ancestral OR7 would give us information about the evolution of this PR.

A Z5-14:OAc receptor still needs to be identified. A candidate PR for this compound is OR30 which is male biased in all four species. The number of substitutions in OR30 is low compared to the number of substitutions found in OR7 orthologues when comparing *C. obliquana/C. herana* and *P. octo/P. excessana* compared to the respective ancestor. One reason could be the higher rate of evolution of OR7, observed for receptors within the pheromone receptor clade (Carraher et al. 2012). Another explanation could be the function of this receptor as a Z5-14:OAc. This compound is used as a sex pheromone in *C. obliquana, C. herana* and *P. excessana* and stabilising selection would prevent many changes in the amino acid sequence. Similar to the suggestions made above it is necessary to repeat transfections of OR30 into HEK293 cells and characterise all OR30 orthologues. In case the functional characterisation is successful it would also be interesting to identify OR30 in all New Zealand native leafroller species. The species within the two genera *Ctenopseustis* and *Planotortrix* use mostly blends consisting of either one or two pheromone components. To have the second PR (the first one would be OR7) identified in the New Zealand native leafroller moths would complete the male side of the equation and contribute largely to the understanding not only of the evolution of sex pheromone reception but would also establish a great basis to finally unravel how mating systems that use chemical systems exactly evolve.
By using single cell recordings there are efforts already underway to test pheromone components on males and females in these species to find out whether females are able to smell their own pheromone components or not.

The understanding of sex pheromone reception at a molecular level by identifying all existing PR candidates and the possibility to produce fertile hybrids opens up the possibility to investigate how traits and preferences for certain pheromone components are inherited. This has been done in *C. obliquana* and *C. herana* before (Hansson et al. 1989), however in the past 15 years technology has advanced hugely and new molecular biology methods are available.

A very interesting project could involve the production of F1, F2 hybrids as well as maternal and paternal backcrosses. Single cell recordings could measure the neuronal activity of male trichoid sensilla. Further wind tunnel experiments could capture the behavioural preference of individuals and using molecular biology tools and functional characterisation of PRs from these hybrids would inform us about the actual binding ability to certain ligands. Maternal backcrosses would be informative to the level of the chromosome only, as there is no recombination in females within Lepidoptera, while paternal backcrosses will be informative within linkage groups. Co-segregation analysis will test for linkage between variation in sex pheromone receptor genes and ability to detect female pheromones as well as if these traits are autosomal or sex-linked.

The characterisation of PRs can also be useful in applied research. *C. obliquana* is a pest in horticulture and methods for mating disruption could be developed using PRs for population control. The concerns about eliminating the polyphagous species in the New Zealand native leafroller species is without reason. Although I can see why it may seem odd to classify a native species as a pest in New Zealand, it has to be mentioned that these species occur widespread throughout the country. The goal is not to drive these species to extinction but to control the population in orchards to minimise the damage dealt to fruits and trees. The polyphagous species within *Ctenopseustis* (*C. obliquana* and *C. herana*) and *Planotortrix* (*P.octo* and *P. excessana*) are common all over New Zealand and it is not intended to decimate these species outside of orchards.

Apart from male biased ORs also female biased ORs could be identified. Unlike male biased ORs, where the expression differences between male and female antennae were congruent in RNAseq-count data and qPCR experiments, the results of these two methods to identify female biased ORs were not consistent for all ORs. However, the ORs that could be identified as female biased in both RNAseq and qPCR can be useful to unravel the mechanism behind close range pheromones. Males in some moth species have been shown to produce sex pheromones emitted during courtship. Since these male sex pheromones are more important for females than for males it could be expected that ORs responding to male sex pheromones are more highly expressed in female than in male antennae. Further female biased ORs could be important to detect plant volatiles important for the female to find...
host plants. The genera *Ctenopseustis* and *Planotortrix* consist of species that are polyphagous as well as monophagous. The investigation of female biased ORs could give information about the evolution of host specificity.

In this PhD study PRs of the New Zealand endemic leafroller sibling species, *C. obliquana*, *C. herana*, *P. octo* and *P. excessana* were identified and characterised to gain more understanding of how sex pheromone perception in male moths evolves. Sexual dimorphism on the antennae was discovered, with s. trichodea type I only present in males. Testing for expression differences of OR genes between male and female antennae revealed two male biased receptors, OR7 and OR30. OR7 could be detected as male biased in all four species and functional characterisation of CoblOR7 and CherOR7 showed that the ability to detect sex pheromone components is dependent on changes in the amino acid sequence of the protein. While receptors important for the detection of female sex pheromones are under strong selection, currently ‘unused’ receptors would be under relaxed constraint and can mutate freely. Changes in the sensitivity and selectivity of pheromone receptors during periods of relaxed constraint might allow a currently ‘unused’ receptor to evolve to be able to perceive a future novel component produced by a variant female. Such changes may contribute to the formation of a new species, perhaps by producing ‘rare males’ that perceive and therefore are able to ‘track’ divergent female pheromones as suggested in the “asymmetric tracking hypothesis” (Phelan 1992).
References


Corcoran JA (2014) The identification of pheromone receptors from the lightbrown apple moth, *Epiphyas postvittana*. The University of Auckland, New Zealand


