Biology
Insect Molecular Biology (2015) **24**(4), 491–501 doi: 10.1111/imb.12176

Molecular basis for odorant receptor tuning: a short C-terminal sequence is necessary and sufficient for selectivity of mosquito Or8

S. R. Hill, S. Majeed* and R. Ignell

Unit of Chemical Ecology, Department of Plant Protection Biology, Swedish University of Agricultural Sciences, Box 102, 230 52 Alnarp, Sweden

Abstract

A birth-and-death evolutionary model for odorant receptor gene repertoires presumes the creation of repertoires with the capacity for high-level diversity and rapid ligand specificity change. This changes the recognised odour space, directly affecting fitness-related behaviours and ultimately affecting adaptation to new environments and resources. The proximate molecular mechanisms underlying the tuning of odorant receptor repertoires, and thus peripheral olfaction, are unclear. In the present study, we report a concrete example of this model of odorant receptor evolution leading to rapid changes in receptor tuning that leave the peripheral neuronal circuitry intact. We identified a conserved odorant receptor gene in mosquitoes, *Or8*, which in *Culex quinquefasciatus* underwent a duplication and inversion event. The paralogues differ in only minor structural changes manifesting at the C-terminus. We assessed the specificity of the paralogous odorant receptors and receptor neurones. We found that the functional tuning of the receptor was indeed reflected in minor differences in amino acid structure. Specifically, we found that enantiomeric specificity of these mosquito *Or8* paralogues relies on eight C-terminal amino acids encoded in the final exon of the gene; thus, the birth of a paralogous odorant receptor can change the tuning of the peripheral olfactory system.

Keywords: olfaction, receptor-ligand interaction, selectivity, birth-and-death evolutionary model, insects, chemical ecology.

Introduction

An animal's fitness largely depends on locating suitable resources and avoiding threats. In the vast majority of animals it is the olfactory system that is at the vanguard of these activities. One way in which olfactory systems can evolve rapidly is through differential selection pressures on the olfactory receptor genes, allowing animals to adapt to various ecological environments (Nei *et al.*, 2008; Hansson & Stensmyr, 2011; Cande *et al.*, 2012). One of the olfactory receptor gene repertoires, the odorant receptors, is predicted to have emerged via a birth-and-death evolutionary model, thereby sharing a common ancestral gene (Nei *et al.*, 2008; Sánchez-Gracia *et al.*, 2009; Hansson & Stensmyr, 2011). This process of gene duplication, which releases one of the genes from current selection pressures to accrue mutations that inactivate or modulate its function, has resulted in relatively large receptor families with highly varying ligand selectivity and sensitivity. The rapid structural and functional modification of olfactory receptors is believed to underpin the versatility of olfactory systems through evolutionary time, while leaving the animal's neuronal circuitry largely intact (Cande *et al.*, 2012), thus probably playing a significant role in adaption and evolution of insects.

The proximate mechanisms underlying odour specificity (for review see Bohbot & Dickens, 2012) are still unknown, while the overall evolutionary mechanisms surrounding the evolution of olfaction have received some attention (Nei *et al.*, 2008; Hansson & Stensmyr, 2011; Cande *et al.*, 2012). One reason for this is that,

First published online 1 June 2015.

Correspondence: Sharon Rose Hill, Unit of Chemical Ecology, Department of Plant Protection Biology, Swedish University of Agricultural Sciences, Box 102, 230 52 Alnarp, Sweden. Tel./fax: +46-40-41 51 05; e-mail: sharon.hill@slu.se

**Current address:* Department, of, Zoology, Government College University, Lahore, Punjab, Pakistan

while information concerning olfactory gene repertoires is growing, we have limited knowledge concerning the links between the odorant receptor gene (*Or*) structure and the odour specificity of its corresponding odorant receptor protein (Or) and olfactory receptor neurone (ORN).

Odorant receptors are putative seven transmembrane domain proteins expressed in the plasma membrane of ORNs of insects as heteromers. A highly conserved chaperone and ion channel, Orco, has been shown to be necessary for the Or complex to be expressed in the membrane (Larsson *et al*., 2004; Sato *et al*., 2008; Wicher *et al*., 2008). The complex also includes a unique Or that contributes to the specificity and sensitivity of the complex (see review Stengl & Funk, 2013). Current models describe this heteromeric complex as a dimer, yet the structure and stoichiometry of the complex is not yet known. What constitutes modulatory domain(s) in these unique Ors, regulating ligand specificity, is unclear. Two recent studies have indicated that minor changes in the unique Or structure, as small as the substitution of a single amino acid, can substantially alter the selectivity of the receptor (Nichols & Luetje, 2010; Leary *et al*., 2012). As the unique Ors are under differential adaptive pressures and can change structure rapidly under selecting conditions, ligand specificity may vary rapidly over evolutionary time; therefore, to determine the extent by which structural variation plays a role in Ors' specificity to ligands, paralogues of unique Ors with varying ligand specificity must be investigated.

In the mosquitoes *Culex quinquefasciatus, Anopheles gambiae* and *Aedes aegypti*, a set of orthologous/paralogous Ors, generally known as the Or8s, have been identified (Hill *et al*., 2002; Bohbot *et al*., 2007; Arensburger *et al*., 2010; Pelletier *et al*., 2010). The Or8s provide an excellent model system to study the mechanism underlying receptor-ligand specificity in the insect olfactory system. Previous studies have established that female mosquitoes display a striking difference in specificity for the enantiomers of 1-octen-3-ol, common vertebrate host-related emanations, at the behavioural (Kline *et al*., 2007; Cook *et al*., 2011), physiological (Syed & Leal, 2007; Ghaninia *et al*., 2008; Cook *et al*., 2011; Grant & Dickens, 2011) and receptor level (Lu *et al*., 2007; Bohbot & Dickens, 2009). At each level, mosquitoes exhibit a higher sensitivity to (*R*)-1-octen-3-ol than to (*S*)-1-octen-3-ol (Lu *et al*., 2007; Syed & Leal, 2007; Bohbot & Dickens, 2009; Cook *et al*., 2011; Grant & Dickens, 2011). It is important to note that, while enantiomers appear similar in structure, they are mirror images of one another, and a receptor responds to each enantiomer as a different ligand; just as a left hand glove fits snugly on the left, but not the right, hand. The amino acid structures of the Or8s have been predicted from the three available mosquito genome databases, and two of the orthologues, *Ae. aegypti* Or8 (AaOr8) and *An. gambiae* Or8 (AgOr8), have been functionally confirmed by heterologous expression in *Xenopus* oocytes (Lu *et al*., 2007; Bohbot & Dickens, 2009; Wang *et al*., 2010) and the fly empty neurone system (Carey *et al*., 2010).

In the present study, we describe the first strong evidence of the birth-and-death evolutionary model as the foundation of the proximate mechanism that underpins odour specificity using the *Or8* expressing neurones *in vivo* and of *Or8*-expressing insect cell lines *in vitro*. This was done in comparison with the putative receptor structure of the paralogous *Or8s* in *C. quinquefasciatus* (*CqOr113* and *CqOr118*) and with the generation of chimeric *Or8s*.

Results

Odorant receptor neurones

The capitate pegs on the maxillary palps of *C. quinquefasciatus* contain three ORNs classified by amplitude of neuronal response as the A cell, which gives the highest amplitude and has the key ligand carbon dioxide, the B cell, which has an intermediate amplitude and has been shown to have (*R*)-1-octen-3-ol as a key ligand, and the C cell with the lowest amplitude, for which a natural key ligand has yet to be discovered (Fig. 1A; Syed & Leal, 2007). Single sensillum recordings from capitate peg sensilla on the maxillary palps of 10 female *C. quinquefasciatus* mosquitoes confirmed that the B cell neurones responded in a dose-dependent manner to both enantiomers of 1-octen-3-ol with different sensitivities (Fig. 1B). Neither the A nor the C cells responded to either enantiomer at any of the doses tested. The (*R*)-1-octen-3-ol enantiomer elicited robust firing in the B cell at 10 ng, 121.6 ± 5 spike/s, while (*S*)-1-octen-3-ol elicited only 55.4 ± 8 spike/s at 10 ng (Fig. 1). To elicit a similar firing rate in the B cell as 10 ng (*S*)-1-octen-3-ol, 100 times less (*R*)-1-octen-3-ol (0.1 ng) is required (50.2 ± 2 spikes/s). As the purity of the (*S*)-1-octen-3-ol used was determined to be $> 99.9\%$, with the remaining contaminants known to contain some (*R*)-1-octen-3-ol, it is likely that the neuronal response elicited by '(*S*)-1-octen-3-ol' in the present study was actually a response to a 0.01% contamination with (*R*)-1-octen-3-ol. The neurones responded to (*R*)-1-octen-3-ol at between 10 and 100-fold higher sensitivity than to (*S*)-1-octen-3-ol ($F_{df3,\ df134} = 27.75$, $P < 0.0001$) with a half maximum effective concentration (EC_{50}) $= 118 \times 10^{-3}$ ng, and $EC_{50} = 13.0 \times 10^{-3}$ ng), respectively.

Odorant receptors

Expression

Initial attempts to differentially amplify *CqOr113* and *CqOr118* from maxillary palps using reverse transcriptase-

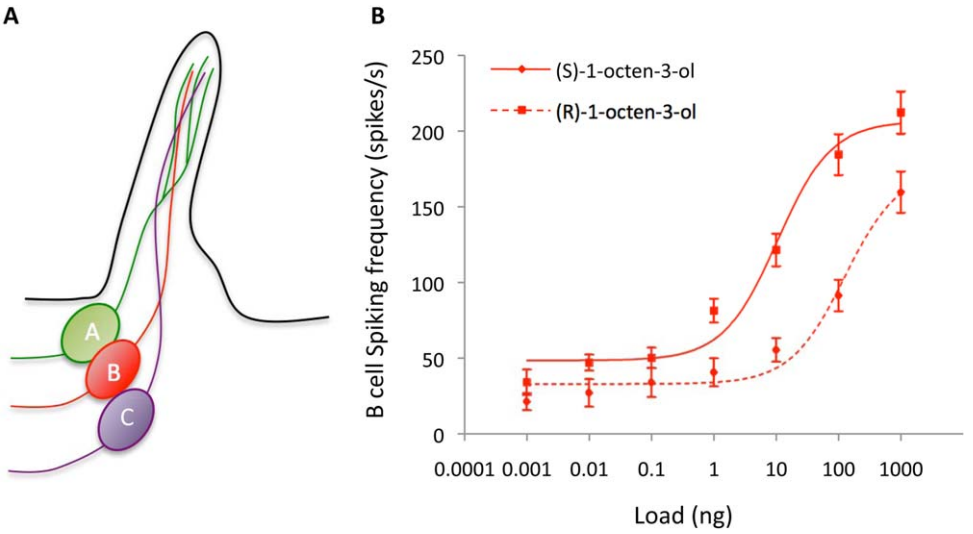

Figure 1. Enantiomeric specificity of 1-octen-3-ol sensitive olfactory receptor neurones. (A) The olfactory receptor neurones in the maxillary palp capitate peg sensillum are classified as the A cell, the B cell and the C cell. (B) Single sensillum recordings from the B cells of 10 female *Culex quinquefasciatus* mosquitoes show that these cells respond differentially to (*R*)-(−)-1-octen-3-ol (squares, solid line) and (*S*)-(+)-1-octen-3-ol (circles, dotted line).

PCR were unreliable, as these paralogues differ only in the final exon (63 nucleotides (nt)/66 nt, and 67%/62% AT-rich, respectively) and paralogue-specific primers were not found. Ongoing transcriptome analyses revealed the presence of reads for both *CqOr118* and *CqOr113*, and thus indicated that both paralogues are expressed in the maxillary palps of female *C. quinquefasciatus* (Fig. 2). A full description and analysis of the *C. quinquefasciatus* maxillary palp transcriptome will be presented elsewhere (S.R., Hill and R., Ignell, unpublished data). Orthologues to the other *Or* and *Grs* previously found to be expressed in the maxillary palps of *Ae. aegypti* (*AaOr49* as well as *AaGr1,*

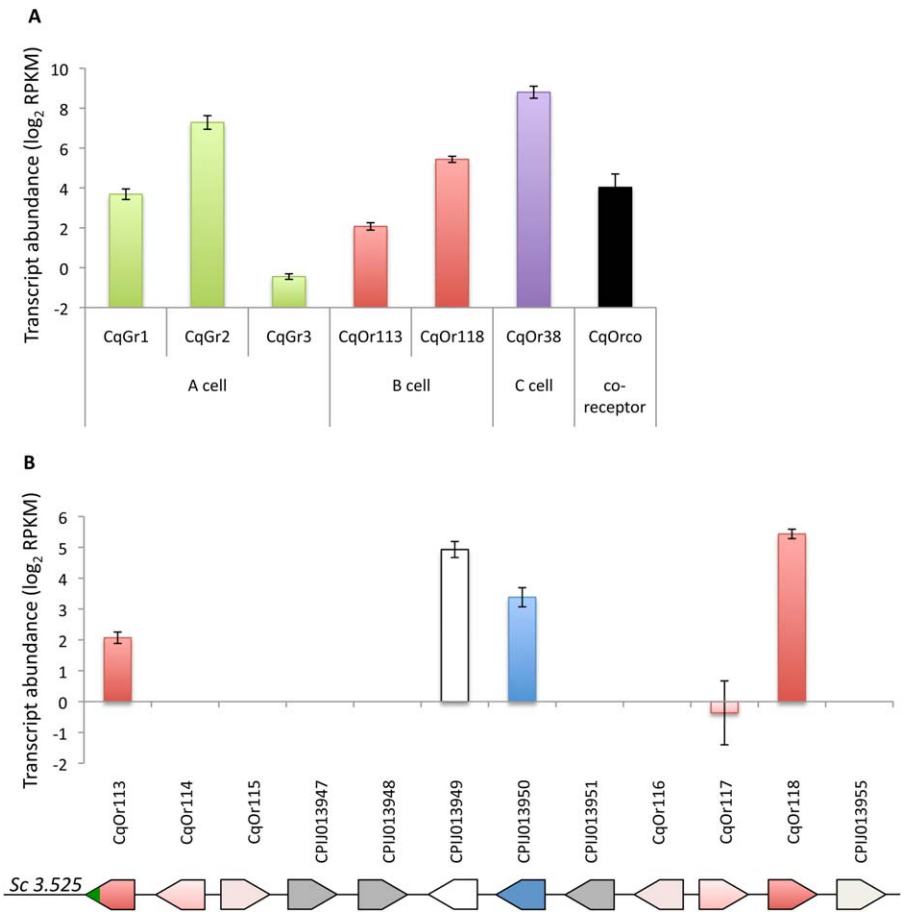

Figure 2. Odorant receptor gene expression in the maxillary palps. (A) The relative transcript abundance of the *Culex quinquefasciatus* orthologues of mosquito maxillary palp genes expressed in the A cell (green), B cell (red) and C cell (purple) is shown, as well as the gene for the canonical odorant receptor, Orco (black). (B) The relative transcript abundance of genes expressed in a cluster on *C. quinquefasciatus* supercontig 3.525 is shown, including the odorant receptor genes *Or113* through *Or118* (red hues). Bottom panel represents the microsynteny with paralogues depicted by the same colour and hue. In particular, *CqOr113* (dark red) is shown as a paralogue of *CqOr118* following a local duplication and inversion event that left *CqOr113* with a new final exon, shown here in green. RPKM, reads per kilobase per million mapped reads.

AaGr2 and *AaGr3*; Bohbot *et al*., 2007) were also found to be expressed in the maxillary palps of *C. quinquefasciatus* (CqOr38 as well as *CqGr1, CqGr2* and *CqGr3*), as was the co-receptor, *CqOrco* (Fig. 2A). The *CqOr8* paralogues are found within 75 kb of one another in a region of the genome (supercontig 3.525) that has undergone a series of both duplication and inversion events (Fig 2B) (Arensburger *et al*., 2010). Of the other *Ors* found in this stretch of genome between the *Or8* paralogues, *CqOr114* and *CqOr117*, also paralogues, were expressed in the maxillary palps in low abundance, whereas the paralogues *CqOr115* and *CqOr116* were not expressed at all (Fig. 2B) indicating that the each pair of *Or* paralogues in this region is under different transcriptional regulation. In a comparison of the 3 kb of sequence upstream of these *Or8* paralogues, the sequence identity is 98.6% over 2494 nt, including three minor ($\leq$10 nt) indels and five indels associated with nucleotide runs ($+$1 or 2 nt), and found in three segments separated by two indels that are 570–779 nt and 851–1202 nt upstream of *CqOr118*. As there is ca. 5 kb separating the *Or8* paralogues from their closest upstream neighbours (*CqOr114* and *CqOr116*), it is likely that the conserved tissue-specific regulatory region for *CqOr8* expression in the maxillary palps falls within this region. A comparison of this region with *Ae. aegypti* and *An. gambiae* revealed no obvious conservation in upstream sequences. Of the genes found between the CqOr8 paralogues that are indicated by VectorBase accession numbers in Fig. 2, all but one (CPIJ013949) are as of yet unidentified and are annotated as conserved hypothetical proteins. CPIJ013949 is annotated as developmentally regulated GTP-binding protein 2 and demonstrated a similar abundance in the maxillary palp transcriptome as *CqOr118*. None of the other genes in this region were represented in the transcriptome.

Structure

Odorant receptors are currently predicted to be inverted seven transmembrane domain proteins with an intracellular amino terminus and extracellular carboxy terminus (Benton *et al*., 2006; Tsitoura *et al*., 2010; Hopf *et al*., 2015). In the present study, we have predicted the transmembrane structure of the CqOr118 and CqOr113 (http://bioinf.cs.ucl.ac.uk/psipred/) and have used TOPO2 (http://www.sacs.ucsf.edu/cgi-bin/open-topo2.py/) to plot a two-dimensional model of CqOr118 with the differences in the CqOr113 sequence mapped on top of it (Fig. 3A). A comparison between the amino acid sequences of the paralogues CqOr118 and CqOr113 showed polymorphisms only at the C-terminus (Fig. 3A, B; Supporting information, Data S1). At the receptor level, this translates into a seven amino acid insertion (Fig. 3A, B, asterisk) and four amino acid substitutions within the predicted seventh transmembrane domain of CqOr113 (Fig. 3B). In addition, there is a deletion of the entire predicted extracellular C-terminus of CqOr113 (Fig. 3A, B). To test the hypothesis that the eight amino acid C-terminal 'tail' is both necessary and sufficient to confer enantiomeric selectivity, constructs were created in which the 'tail' was removed from CqOr118 and was added to the C-terminus of CqOr113 (Fig. 3C, D). The functional assays of these constructs are described below. An examination of the gene sequence of *CqOr113* also revealed that the final exon that codes for the 20 C-terminal amino acids is neither orthologous with that of *CqOr118* nor with those of the other known mosquito *Or8s* (Fig. 4). A comparison among the C-termini of all four mosquito Or8s revealed strong similarities among CqOr118, AgOr8 and AaOr8 (Fig. 5). Fifteen of the final C-terminal 21 amino acids are identical, and all six substitutions are conservative (Fig. 5). A nucleotide comparison of the final exons of *CqOr118*, *AgOr8* and *AaOr8* revealed a 48% identity, which decreased to 19% with the addition of *CqOr113* to the alignment. A closer examination revealed the final exon of *CqOr113* to be found within what appears to be the 5′ untranslated region (UTR) of the degraded 5′ end of a type II class of transposable element, a P-element type, which includes a terminal inverted repeat, 5′UTR and $\sim$60 nt of the first open reading frame (80.7% identity; 3 indels $\leq$ 10 nt). Together, this suggests that CqOr118 retains the more ancestral structure of the Or8 c-terminus when compared with CqOr113 (Figs. 4 and 5).

Function

To assess the selectivity of the CqOr8 paralogues, CqOr118 and CqOr113 were cloned into an expression vector (pIB) and transfected into Sf9 cells. Calcium imaging of *CqOr8*-transformed Sf9 cells from 3–4 wells across 3–4 plates for each dose showed that the dose–response curve for CqOr118 transfected cells to (*R*)-1-octen-3-ol had a higher specificity than that to (*S*)-1-octen-3-ol ($F_{df3,\ df398} = 31.81$, $P < 0.0001$; Fig. 6A). The calculated EC_{50} for CqOr118 responding to (*R*)-1-octen-3-ol and (*S*)-1-octen-3-ol was 1.43×10^{-3} M and 5.73×10^{-3} M, respectively. In contrast, the *CqOr113*-transformed Sf9 cells did not differentiate in specificity between the enantiomers ($F_{df3,\ df398} = 1.853$, $P = 0.1370$; Fig. 6B). The calculated shared EC_{50} for the CqOr113 response to both enantiomers was $3.02\text{x}10^{-3}$ M. It is important to remember that while it is informative to compare the relative dose-response characteristics of these Ors *in vitro* to determine differences in selectivity, the absolute characteristics from *in vitro* expression assays should not be taken as to apply to the endogenous receptor function.

Structure/function relationship

According to the Or8 receptor modelling (Fig. 3), the most notable difference between the paralogues is in the extracellular C-terminal amino acid 'tail' (TIYDPSEK),

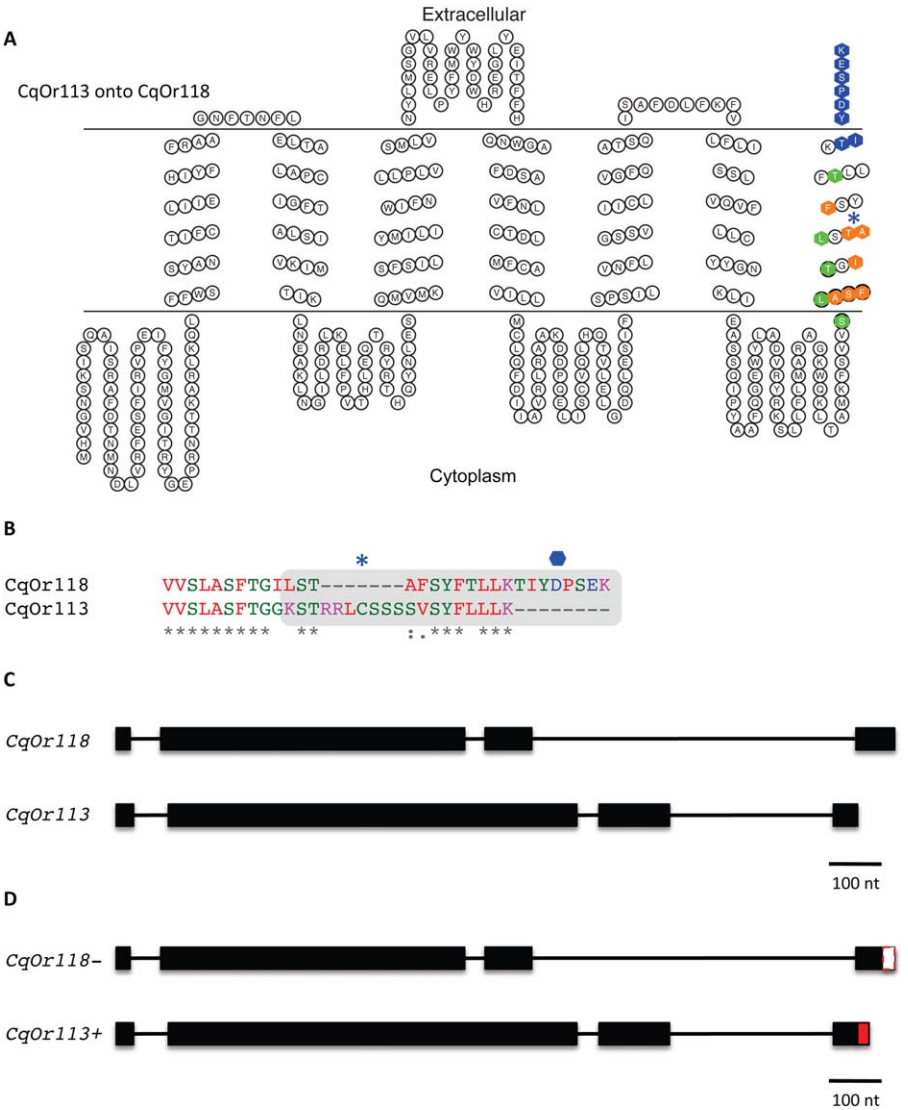

Figure 3. Putative protein structural differences between the enantiomer selective and non-selective *Or8* paralogues. (A) A model of CqOr118 protein orientation and transmembrane domains (TMDs). Amino acids differing from CqOr113 are indicated by colour: conserved substitutions (orange), non-conserved substitutions (green) and missing (blue and *). (B) ClustalO alignment of TMD 7 and the C-termini of the paralogues. The final exon encodes the amino acids in grey. (C) Exon/intron map of the paralogues. (D) Map of chimeric receptors *CqOr118*− and *CqOr113*+: the dashed line indicates the missing *CqOr118* eight amino acids, while the red box encloses their addition to *CqOr113*+.

which is present in the selective CqOr118 and absent in the non-selective CqOr113. To test whether these eight amino acids are both necessary and sufficient to account for the difference in ligand selectivity between the paralogues, we created two mutant *Or8*s:

1. *CqOr118*− — based on the selective *Or8* (*CqOr118*) but missing its C-terminal 'tail' (Figs 3C and 6C); and
2. *CqOr113*+ — based on the non-selective *Or8* (*CqOr113*) with the C-terminal 'tail' added (Figs 3D and 6D).

The deletion of the predicted extracellular C-terminal amino acids from the selective Or8, rendered the chimeric receptor CqOr118- non-selective for either (*R*)- or (*S*)-1-octen-3-ol ($EC_{50} = 9.96 \times 10^{-3}$ M, $F_{df3,\ df1479} = 21.17$, $P < 0.0001$; Fig. 6C) determined from a screen of individual cells from 3–4 wells across 3–4 plates for each dose. This shows that the C-terminal 'tail' is necessary for the enantiomeric selectivity of this Or. By contrast, the addition of the C-terminal 'tail' to the non-selective CqOr113 rendered CqOr113+ selective. CqOr113+ showed a higher specificity of response for (*R*)-1-octen-3-ol ($EC_{50} = 1.32 \times 10^{-3}$ M) over (*S*)-1-octen-3-ol ($EC_{50} = 7.10 \times 10^{-3}$ M, $F_{df3,\ df519} = 52.37$, $P < 0.0001$; Fig. 6D) similar to that of CqOr118 ($EC_{50} = 1.43 \times 10^{-3}$ M, $F_{df3,\ df398} = 31.81$, $P < 0.0001$; Fig. 6A), showing that this region is sufficient to confer the enantiomeric selectivity to this Or.

Discussion

In the present study, we describe a proximate mechanism that underpins odour specificity, and provides concrete functional support for the birth-and-death model of receptor evolution. Minimal changes in the primary structure of an odorant receptor and its gene are shown to have profound effects on the functional properties of the

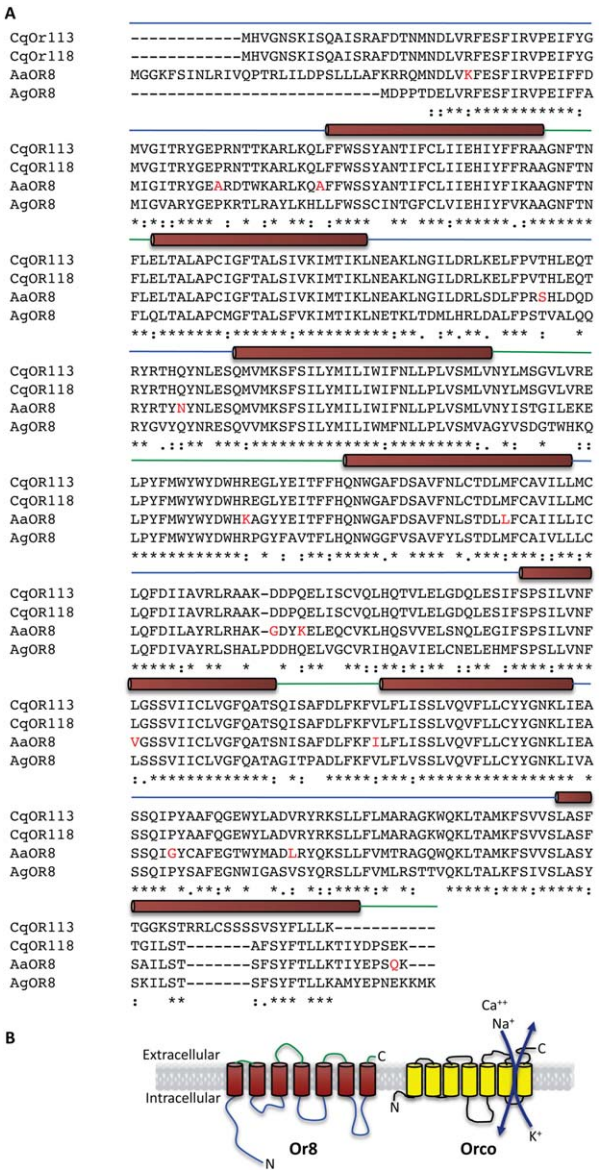

Figure 4. Overall protein structure and similarities among mosquito Or8 orthologues. (A) Amino acid sequence differences among the mosquito Or8s are indicated as highly (:) and moderately (.) conservative substitutions, as well as blanks for non-conservative substitutions. Asterisks (*) indicate identity across all four Or8s. Three characteristics of the predicted protein structure are described: intracellular (green lines), extracellular (blue lines) and transmembrane (dark red boxes) domains. (B) A representation of the Or8 - Orco complex in the plasma membrane.

receptor. At the level of the gene, a duplication and inversion event has lead to the incorporation of a different final exon in the new paralogous receptor gene. In practical terms, this has led to the new receptor lacking the eight extracellular amino acids of the C-terminal 'tail' shown to confer enantiomeric selectivity to this receptor. The overall specificity of the cognate ORN reflects the functional characteristics expected from the demonstrated expression of both paralogues. While it is possible that CqOr113 is now expressed in one of the other two ORNs expressed in the maxillary palps, neither of these ORNs responded to either enantiomer of 1-octen-3-ol. Additionally, the duplication and inversion event that gave rise to the birth of *CqOr113* maintained the paralogue in close proximity with *CqOr118* and did not translocate *CqOr113* downstream of any of the other chemoreceptors known to be expressed in the A cell (*C. quinquefasciatus gustatory receptors 1, 2* and *3*) or C cell (*CqOr38*). There is also strong evidence of conserved sequence upstream of *CqOr118* and *CqOr113*. Also, the combination of microsynteny surrounding *CqOr113* and *CqOr118* with the transcriptome results (Fig. 2) indicates that the expression of both paralogues appears to be regulated independently of those genes located in their immediate upstream region. These data provide evidence to support the hypothesis from Cande *et al*. (2012) that physical and functional changes in chemoreceptors form the basis of the variability in the olfactory system over evolutionary time, without requiring large changes in neuronal circuitry. This implies that insect adaption and evolution may be strongly influenced by a rapidly varying odorant receptor repertoire, especially as the success of many fitness-determining behaviours, including host, mate and oviposition-site preference, are dependent on olfaction.

Selectivity of insect ORNs and Ors has been investigated previously, generally in the context of overall neuronal and receptor tuning, with panels of both structurally related and structurally diverse compounds (Hallem *et al*., 2004; Carey *et al*., 2010; Wang *et al*., 2010; Bohbot & Dickens, 2012). The aim of these studies was to better describe the Or and ORN odour space coverage in the context of the combinatorial coding of the insect olfactory system. As our focus was constrained to the proximate mechanism underlying selectivity at the receptor level, we chose to use a model system comprised of paralogous Ors that respond differentially to the enantiomers of 1-octen-3-ol.

Previous studies have identified the 1-octen-3-ol sensitive ORN in the maxillary palp capitate peg of mosquitoes and determined its sensitivity and enantiomeric selectivity (Lu *et al*., 2007; Syed & Leal, 2007; Cook *et al*., 2011; Grant & Dickens, 2011). In addition, the corresponding *Ors* in *Ae. aegypti* (Bohbot & Dickens, 2009), *An. gambiae* (Lu *et al*., 2007) and *C. quinquefasciatus* (present study) have been identified. Heterologous expression of *AaOr8* (Lu *et al*., 2007), *AgOr8* (Bohbot & Dickens, 2009) and *CqOr118* (present study) have showed that the threshold of response to (*R*)-1-octen-3-ol is significantly lower than that for (*S*)-1-octen-3-ol (Lu *et al*., 2007; Bohbot & Dickens, 2009; present study). In contrast, we found no difference in the response generated by stimulation with either enantiomer of 1-octen-3-ol in Sf9 cells

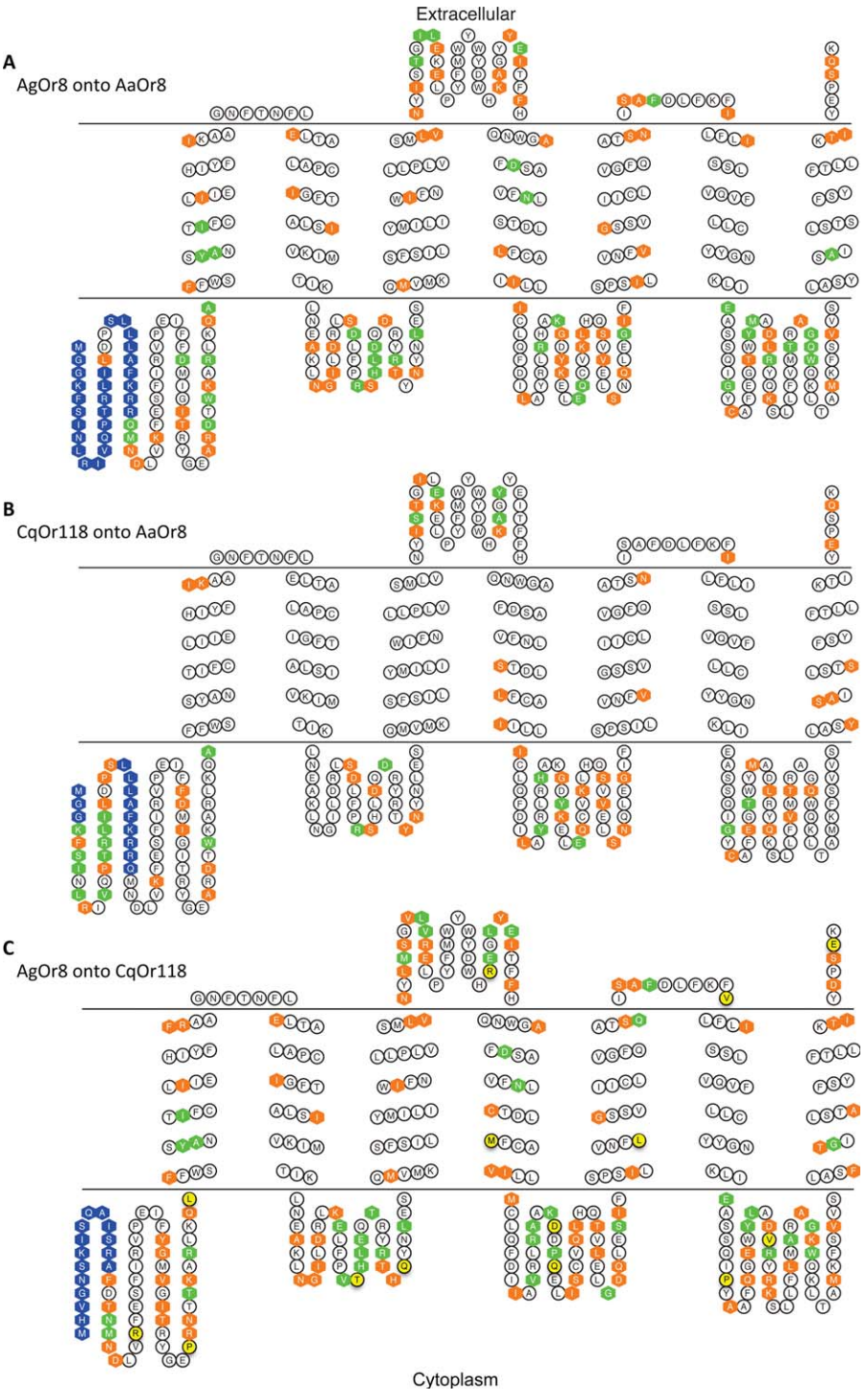

Figure 5. Predicted protein structural model indicating differences between pairs of mosquito Or8 orthologues. (A) *Anopheles gambiae* AgOr8 is mapped onto *Aedes aegypti* AaOr8; (B) *Culex quinquefasciatus* CqOr118 is mapped onto the *Ae. aegypti* AaOr8; and (C) *An. gambiae* AgOr8 is mapped onto *C. quinquefasciatus* CqOr118. The sequence-based models show protein orientation and transmembrane domains. Colours indicate conserved substitutions (orange), non-conserved substitutions (green) and deletions (blue). Amino acids identical between the *R*-(−)-1-octen-3-ol high-sensitivity Or8s (AgOr8 and CqOr118) but differ from the lower sensitivity Or8 (AaOr8) are coloured yellow (bottom panel). Two-dimensional representations created using TOPO2 software (Johns SJ, TOPO2, Transmembrane protein display software, http://www.sacs.ucsf.edu/TOPO2/).

expressing *CqOr113*. A comparison between the gene structures of the *C. quinquefasciatus Or8* paralogues showed no differences in the amino acid coding except in the final exon of *CqOr113*. While this exon has *Or* characteristics, it is not directly related to the final exon of *CqOr118*, *AaOr8* or *AgOr8*, and appears to be derived from the degraded footprint of transposable element activity. From this, we hypothesise that the putative extracellular C-terminal region of the Or8s may be essential for generating the specificity of the receptor for the enantiomers of 1-octen-3-ol. Several previous studies have investigated the ability of various insects to detect ecologically relevant enantiomers (e.g. Laska & Galizia, 2001; Ulland *et al.*, 2006; Lu *et al.*, 2007; Bohbot &

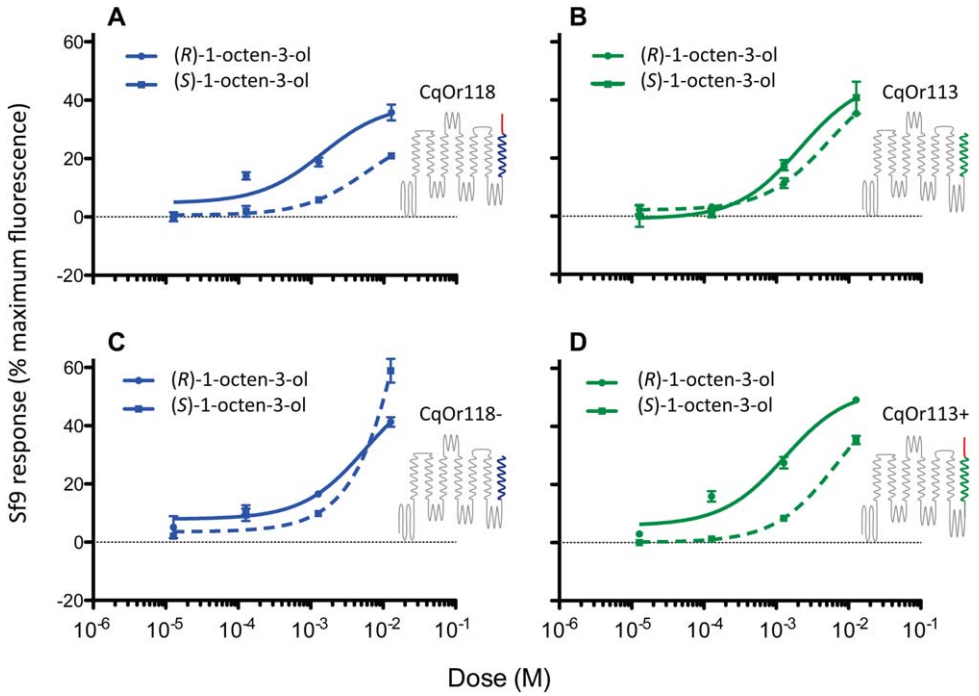

Figure 6. Enantiomeric specificity of paralogous and chimeric Or8s expressed in an insect cell line. The specificity for *R*-(+)-1-octen-3-ol (solid lines) and *S*-(−)-1-octen-3-ol (dashed lines) of the heterologously expressed Or8s, CqOr118 (A) and its paralogue CqOr113 (B); as well as the chimeric CqOr118−, missing the extracellular C-terminal 'tail' (C), and Or113+, with the extracellular C-terminal 'tail' added (D). Screens of individual cells from 3–4 wells across 3–4 plates were conducted at each dose. Statistical significance was found between the intensity of fluorescent responses from *S*-(+)-1-octen-3-ol and *R*-(−)-1-octen-3-ol as determined by non-linear regression analyses in (A) and (D) ($P > 0.0001$).

Dickens, 2009; Cook *et al*., 2011); however, this is the first study in which these behavioural and physiological differences have been correlated with the structure of their cognate receptors. While differential enantiomeric selectivity of CqOr118 and CqOr113 has been demonstrated, a natural ligand for the duplicated paralogue CqOr113 has not yet been found. For a confirmation the birth-death model for receptor evolution, it is required to show that evolutionary forces act on newly born receptors leading to the ultimate death or sustained existence of the receptor in the genome of a specific species/population. In the present study, we have shown that the accumulation of SNPs in the duplicated *CqOr113* (seven SNPs, ignoring the final exon) is similar to those accumulated by the neighbouring Or gene paralogues also involved in the original duplication and inversion event (four SNPs between *CqOr114* & *CqOr117* and eight SNPs between *CqOr115* & *CqOr116*) (Arensburger *et al*., 2010); therefore, the mutation rate for *CqOr113* is not higher than that of its neighbours, indicating that evolutionary forces are acting equally on all of the Ors in this region, and suggesting a continued functional role for *CqOr113*. Whether CqOr113 has acquired a separate functional role in the overall selectivity of the B cell in addition to its contribution to the regulation of the B cell's (*R*)-1-octen-3-ol sensitivity is unknown at this time.

In a recent study, Leary *et al*. (2012) showed that a single amino acid difference in the predicted third transmembrane domain narrowed the selectivity of an odorant receptor in the European corn borer, Or3, from responding to (*E*)-12- and (*Z*)-12-tetradecenyl acetate as well as (*E*)-11- and (*Z*)-11-tetradecenyl acetate, to only to (*E*)-12- and (*Z*)-12-tetradecenyl acetate (Leary *et al*., 2012). In this case, the change in sexual pheromone specificity of the receptors provides a clear mechanism for generating reproductive isolation, which in turn can lead to speciation. The findings in this study describe the change in the specificity of a class of odorant receptors expressed in maxillary palps, the *Or8s*, that have been described as highly important in the host-seeking and host choice of mosquitoes (Lu *et al*., 2007; Bohbot & Dickens, 2009; Grant & Dickens, 2011; Cook *et al*., 2011). The ligand of the Or8 orthologues in *Ae. aegypti* (AaOr8; Bohbot & Dickens, 2009; Grant & Dickens, 2011), *An. gambiae* (AgOr8; Lu *et al*., 2007), and *C. quinquefasciatus* CqOr118; (present study) is (*R*)-1-octen-3-ol, a volatile compound that is relevant to mosquito host choice, as is present in the breath and skin emanations of mammals, yet absent from the emissions of other potential hosts, such as birds (Kline *et al*., 2007). In fact, both Or8 and their cognate maxillary palp neurones have narrowly tuned ligand specificities in *Ae. aegypti* and *An. gambiae* (Lu *et al*., 2007; Bohbot & Dickens, 2009; Grant & Dickens, 2011), as do the maxillary palp neurones in *C. quinquefasciatus* (Syed & Leal, 2007). Together with carbon dioxide, (*R*)-1-octen-3-ol has been described as a host recognition cue mediating attraction to the host in *Ae. aegypti* (Cook *et al*., 2011), whereas in *C. quinquefasciatus* (*R*)-1-octen-3-ol has been shown to be repellent at amounts common in the breath of large mammals (Cook *et al*., 2011). Such changes in host choice in mosquitoes have the potential

to affect both offspring fitness and mate choice. Mosquito mating in many species has been shown to be associated with the preferred host (Hartberg, 1971; McIver, 1980) and thus, host choice may directly affect mate choice. Also, the fecundity of females varies significantly depending on the different composition of blood from a variety of host animals (Ikeshoji, 1964; Richards *et al.*, 2012). The findings from the present study, together with those from Leary *et al.* (2012), show that discrete mutations that change the specificity of odorant receptors may provide a mechanism that contributes to reproductive isolation.

Minor structural variations in Or genes, which have accumulated through evolutionary time, give rise to differences in receptor tuning. The duplication of Or genes, releasing one paralogue from the current selection pressures, creates favourable conditions for the diversification of function within the Or repertoire. The accumulation of such polymorphisms throughout the Or gene repertoire appears to act as the mechanism by which broad variations can be generated in the odour space of individuals within a species. Indeed, it may form the basis for interspecies differences in resource and niche preference.

Experimental procedures

Animals

Culex quinquefasciatus (Thai strain) were reared at 27 ± 1°C, 65 ± 5% relative humidity under a 12 h:12 h light:dark period, as previously described (Cook *et al.*, 2011). For all experiments, 4- to 10-day post-emergence sugar-fed adult female mosquitoes were used, unless otherwise stated.

Electrophysiology

The capitate peg sensilla cover the fourth segment of maxillary palps of female *C. quinquefasciatus*. Each sensillum contains three ORNs (McIver *et al.*, 1972). The intermediate spiking amplitude neurone, by convention referred to as the B cell, responds to 1-octen-3-ol (Lu *et al.*, 2007; Syed & Leal, 2007; Cook *et al.*, 2011). Electrophysiological recordings from these neurones were made using single sensillum recording as described previously (Cook *et al.*, 2011). The B cell was stimulated by introducing the enantiomers of 1-octen-3-ol into the humid airstream passing over the preparation 11 cm upstream of the maxillary palps. (*R*)-(−)-1-octen-3-ol (99.6% R) and (*S*)-(+)-1-octen-3-ol (99.9% S) were a gift from Dr James Logan (London School of Hygiene & Tropical Medicine, UK).

Expression of 1-octen-3-ol receptors *in vivo*

As a result of the very high nucleotide sequence conservation between the paralogous receptors (99.3% identical 1-1167 nt; and 27.7% identical over the final exon 1168–1233 nt), it was not possible to consistently discriminate between paralogues using PCR. This was not, however, a problem using next-generation sequencing. Maxillary palps (500) were dissected from 5- to 6-day post-emergence female *C. quinquefasciatus*, which had *ad libitum* access to 10% sucrose and had not been blood fed. Tissues were stored at −20°C in RNAlater (Life Technologies, Stockholm, Sweden). Total RNA was extracted from maxillary palps using the RNeasy Kit (Qiagen, Sollentuna, Sweden) and then stored for ≤2 months at −80°C. Total RNA was quantified using the fluorometric analysis (Qubit; Invitrogen, Stockholm, Sweden) and quality assessed by NanoDrop and standard gel electrophoresis prior to sending to Eurofins (Ebersberg, Germany) for 3′ fragment cDNA library generation and sequencing on Illumina HiSeq 2000. Two bar coded fragment libraries (technical replicates) were generated from 500 ng total RNA each following the standard Illumina protocol. Sequencing was carried out on an Illumina HiSeq 2000 (single end read, 1×100 bp; 32 013 476 reads total for the maxillary palp library).

Prior to mapping, the raw reads were analysed and quality filtered. The adapter sequences and bad quality bases (Phred score < 20) were removed from both ends of each single read. Reads that did not fulfil an average quality threshold were clipped (sliding window, window size 20 bp, minimum quality 4). Finally, reads <40 bp were removed. The remaining high quality reads were mapped to the *C. quinquefasciatus* Johannesburg transcriptome (VectorBase; https://www.vectorbase.org/download/culex-quinquefasciatus-johannesburgtranscriptscpipj22fagz) by using BWA (http://bio-bwa.sourceforge.net; Li & Durbin, 2009), SamTools (http://samtools.sourceforge.net; Li *et al.*, 2009) and Picard (http://picard.sourceforge.net) Of the total number of high quality reads, 17 405 640 reads (54.4%) mapped to the reference transcriptome. This level of mapping is consistent with other *C. quinquefasciatus* transcriptomes (e.g. Leal *et al.*, 2013). Abundance of reads per transcript is presented as reads per kilobase per million reads (RPKM).

Expression of 1-octen-3-ol receptors in insect cells

Full-length coding sequences of *C. quinquefasciatus Or113* and *Or118* genes (Arensburger *et al.*, 2010) as well as the chimeric sequences *Or113*+ and *Or118*− were synthesised by GenScript USA Inc. (Piscataway, NJ, USA), then cloned individually into the pIB/V5His vector (Invitrogen, Carlsbad, CA, USA). The *Or8s* were expressed in the *Spodoptera frugiperda* 9 (Sf9) cell line (Invitrogen) as described by Kiely *et al.*, (2007). The Sf9 cell line is a heterogeneous cell culture, which includes cells that naturally express *S. frugiperda* Orco (confirmed by reverse transcriptase-PCR). Briefly, the transfection agent, Escort IV (Sigma Aldrich, Stockholm, Sweden), was added along with the purified plasmid DNA to Sf9 cells and transfection took place during a 7 h incubation at 28°C. Transfected cells were washed and then incubated for 48 h in fresh media prior to calcium imaging.

Calcium imaging

An inverted Nikon microscope (Eclipse Ti-U, Nikon Co, Japan) enabled fast automatic screening of calcium image captures from 12-well plates, thus providing a moderate throughput assay of the odorant receptors. Transformed Sf9 cell lines were placed in individual wells, loaded with Ca^{2+} sensitive dye

(Fluo4; Life Technologies) in assay saline (21 mM potassium chloride, 12 mM sodium chloride, 18 mM magnesium chloride, 3 mM calcium chloride dihydrate, 170 mM D-glucose, 1 mM probenecid (Sigma-Aldrich), 10 mM PIPES dipotassium salt) and screened for response to the enantiomers of 1-octen-3-ol. Untransfected cells were also assayed with both enantiomers of 1-octen-3-ol, which verified that there was a lack of innate calcium influx in response to the test compounds. The calcium imaging screening process followed that described by Kiely *et al*. (2007). Briefly, a region of the well was randomly selected for imaging. During the first cycle six images were taken at 10-s intervals with no stimulus added. Following the addition of each solution, six more images were taken at 10-s intervals, resulting in four 1-min cycles and a total of 24 images for analysis. At the beginning of the second cycle, assay saline (50 μl) was added next to verify that the cells did not respond indiscriminately to the addition of any solution. The third cycle started with the addition of one dose of the test compound (50 μl) and 1 min later the fourth cycle followed with a stimulation of maximal fluorescence in all Sf9 cells in that well using ionomycin (2.5 μg in 50 μl). Each well was screened individually and image settings were established before the well was assayed and were held consistent throughout the screening of the well. Response of each individual cell to the test compound was determined as the relative change in fluorescence (%) averaged response across all six images between the averaged response to the saline control (0%) and the averaged maximum response to ionomycin (100%).

Molecular structural modelling

Two main algorithms, MEMSAT3 and MEMSAT-SVM, were used to predict the transmembrane domains in the mosquito Or8s using the web-based services at Bloomsbury Centre for Bioinformatics, University of California, Los Angeles (http://bioinf.cs.ucl.ac.uk/psipred/). The two-dimensional model for each of the Ors was plotted using TOPO2 (http://www.sacs.ucsf.edu/cgi-bin/open-topo2.py/).

Data analysis

Non-linear regression analyses were used to analyse the *in vivo* and *in vitro* physiological data using the standard slope model as a reasonable estimate since we report the dose response curve for a limited number of doses (GraphPad Prism version 5.0f for Mac OS X, GraphPad Software, San Diego, CA, USA). We used the extra sum-of-squares F test, thereby presenting the F ratio (F) with its concomitant degrees of freedom ($Df_{numerator}/Df_{denominator}$) and calculated *P* value. In the present study, the numerator of the F ratio is the stimulus dose and the denominator is the number of responding cells. Each individual cell in a single well was treated as an individual replicate. The variation in cell responses among wells and among plates were assessed and found to meet the criteria for treating all cells as a single population. At least three wells on each plate were used for each concentration and at least three plates were run for each construct to control for potential well/day effects. The results of the F test were considered significantly different at $P < 0.05$.

Acknowledgements

We thank Richard Newcomb, Pablo German and Colm Carraher at Plant and Food Research, Mt. Albert campus, Auckland New Zealand for training in the heterologous expression of Ors in Sf9 cells. We also thank James Logan at the London School of Hygiene & Tropical Medicine for his gift of the enantiomers of 1-octen-3-ol. This work was funded by the Linnaeus initiative 'Insect Chemical Ecology, Ethology and Evolution' IC-E^3 (The Swedish Research Council Formas; Swedish University of Agricultural Sciences).

References

Arensburger, P., Megy, K., Waterhouse, R.M., Abrudan, J., Amedeo, P., Antelo, B. *et al*. (2010) Sequencing of *Culex quinquefasciatus* establishes a platform for mosquito comparative genomics. *Science* **330**: 86–88.

Benton, R., Sachse, S., Michnick Stephen, W.S.W. and Vosshall Leslie, B.L.B. (2006) Atypical membrane topology and heteromeric function of Drosophila odorant receptors in vivo. *Plos One* **4**: e20.

Bohbot, J.D. and Dickens, J.C. (2009) Characterization of an enantioselective odourant receptor in the yellow fever mosquito *Aedes aegypti*. *PLoS One* **4**: e7032.

Bohbot, J.D. and Dickens, J.C. (2012) Selectivity of odourant receptors in insects. *Front Cell Neurosci* **6**:29.

Bohbot, J.D., Pitts, R.J., Kwon, H.W., Rutzler, M., Robertson, H.M. and Zwiebel, L.J. (2007) Molecular characterization of the *Aedes aegypti* odorant receptor gene family. *Insect Mol Biol* **16**: 525–537.

Cande, J., Prud'homme, B. and Gompel, N. (2012) Smells like evolution: the role of chemoreceptor evolution in behavioral change. *Curr Opin Neurobiol* **23**: 152–158.

Carey, A.F., Wang, G., Su, C.Y., Zwiebel, L.J. and Carlson, J.R. (2010) Odorant reception in the malaria mosquito *Anopheles gambiae*. *Nature* **464**: 66–71.

Cook, J.I., Majeed, S., Birkett, M.A., Ignell, R., Logan, J.G. and Pickett, J.A. (2011) Enantiomeric selectivity in behavioural and electrophysiological responses of *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes. *Bull Entomol Res* **101**: 541–550.

Ghaninia, M., Larsson, M., Hansson, B.S. and Ignell, R. (2008) Natural odor ligands for olfactory receptor neurons of the female mosquito *Aedes aegypti*: use of gas chromatography-linked single sensillum recordings. *J Exp Biol* **211**: 3020–3027.

Grant, A.J. and Dickens, J.C. (2011) Functional characterization of the octenol receptor neuron on the maxillary palps of the yellow fever mosquito, *Aedes aegypti*. *PLoS One* **6**: e21785.

Hallem, E.A., Ho, M.G. and Carlson, J.R. (2004) The molecular basis of odor coding in the Drosophila antenna. *Cell* **117**: 965–979.

Hansson, B.S. and Stensmyr, M.C. (2011) Evolution of insect olfaction. *Neuron* **72**: 698–711.

Hartberg, W. (1971) Observations on the mating behaviour of *Aedes aegypti* in nature. *Bull World Health Organ* **45**: 847–850.

Hill, C.A., Fox, A.N., Pitts, R.J., Kent, L.B., Tan, P.L., Chrystal, M.A. *et al*. (2002) G protein-coupled receptors in *Anopheles gambiae*. *Science* **298**: 176–178.

Hopf, T.A., Morinaga, S., Ihara, S., Touhara, K., Marks, D.S. and Benton, R. (2015) Amino acid coevolution reveals three-dimensional structure and functional domains of insect odorant receptors. *Nat Commun* **6**: 6077.

Ikeshoji, T. (1964) Fecundity of Culex pipiens fatigans Wied. fed on various amounts of blood and different hosts. World Health Organization report 133.65.

Kiely, A., Authier, A., Kralicek, A.V., Warr, C.G. and Newcomb, R.D. (2007) Functional analysis of a *Drosophila melanogaster* olfactory receptor expressed in Sf9 cells. *J Neurosci Methods* **159**: 189–194.

Kline, D.L., Allan, S.A., Bernier, U.R. and Welch C.H. (2007) Evaluation of the enantiomers of 1-octen-3-ol and 1-octyn-3-ol as attractants for mosquitoes associated with a freshwater swamp in Florida, U.S.A. *Med Vet Entomol* **21**: 323–331.

Larsson, M.C., Domingos, A.I., Jones, W.D., Chiappe, M.E., Amrein, H. and Vosshall, L.B. (2004) Or83b encodes a broadly expressed odourant receptor essential for Drosophila olfaction. *Neuron* **43**: 703–714.

Laska, M. and Galizia, C.G. (2001) Enantioselectivity of odor perception in honeybees (*Apis mellifera* Carnica). *Behav Neurosci* **115**: 632–639.

Leal, W.S., Choo, Y.M., Xu, P., da Silva, C.S. and Ueira-Vieira, C. (2013) Differential expression of olfactory genes in the southern house mosquito and insights into unique odorant receptor gene isoforms. *Proc Natl Acad Sci USA* **110**: 18704–18709.

Leary, G.P., Allen, J.E., Bunger, P.L., Luginbill, J.B., Linn, C.E., Irene, E. *et al*. (2012) Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *PNAS* **109**: 14081–14086.

Li, H. and Durbin, R. (2009) Fast and accurate short read alignment with Burrows-Wheeler Transform. *Bioinformatics* **25**: 1754–1760.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N. *et al*. (2009) The Sequence alignment/map (SAM) format and SAMtools. *Bioinformatics* **25**: 2078–2079.

Lu, T., Qiu, Y.T., Wang, G., Kwon, J.Y., Rutzler, M., Kwon, H.W. *et al*. (2007) Odor coding in the maxillary palp of the malaria vector mosquito *Anopheles gambiae*. *Current Biol* **17**: 1533–1544.

McIver, S.B. (1972) Fine structure of pegs on the palps of female culicine mosquitoes. *Can J Zool* **50**: 571–576.

McIver, S.B. (1980) Sensory aspects of mate-finding behavior in male mosquitoes (Diptera: Culicidae). *J Med Entomol* **17**: 54–57.

Nei, M., Niimura, Y. and Nozawa, M. (2008) The evolution of animal chemosensory receptor gene repertoires: roles of chance and necessity. *Nat Rev Genet* **9**:951–963.

Nichols, A.S. and Luetje, C.W. (2010) Transmembrane segment 3 of *Drosophila melanogaster* odorant receptor subunit 85b contributes to ligand-receptor interactions. *J Biol Chem* **285**: 11854–11862.

Pelletier, J., Hughes, D.T., Luetje, C.W. and Leal, W.S. (2010) An odorant receptor from the southern house mosquito *Culex pipiens quinquefasciatus sensitive* to oviposition attractants. *PLoS ONE* **5**: e10090.

Richards, S.L., Anderson, S.L. and Yost, S.A. (2012) Effects of blood meal source on the reproduction of *Culex pipiens quinquefasciatus* (Diptera: Culicidae). *J Vector Ecol* **37**: 1–7.

Sánchez-Gracia, A., Vieira, F.G. and Rozas, J. (2009) Molecular evolution of the major chemosensory gene families in insects. *Heredity* **103**: 208–216.

Sato, K., Pellegrino, M., Nakagawa, T., Vosshall, L.B. and Touhara K. (2008) Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature* **452**: 1002–1006.

Stengl, M. and Funk, N.W. (2013) The role of the coreceptor Orco in insect olfactory transduction. *J Comp Physiol A* **199**: 897–909.

Syed, Z. and Leal, W.S. (2007) Maxillary palps are broad spectrum odourant detectors in *Culex quinquefasciatus*. *Chem Senses* **32**: 727–738.

Tsitoura, P., Andronopoulou, E., Tsikou, D., Agalou, A., Papakonstantinou, M.P., Kotzia, G.A. *et al*. (2010) Expression and membrane topology of *Anopheles gambiae* odorant receptors in lepidopteran insect cells. *PLoS One* **5**: e15428.

Ulland, S., Ian, E., Borg-Karlson, A.K. and Mustaparta, H. (2006) Discrimination between enantiomers of linalool by olfactory receptor neurons in the cabbage moth *Mamestra brassicae* (L.). *Chem Senses* **31**: 325–334.

Wang, G., Carey, A.F., Carlson, J.R. and Zwiebel, L.J. (2010) Molecular basis of odor coding in the malaria vector mosquito *Anopheles gambiae*. *Proc Natl Acad Sci USA* **107**: 4418–4423.

Wicher, D., Schäfer, R., Bauernfeind, R., Stensmyr, M.C., Heller, R., Heinemann, S.H. *et al*. (2008) Drosophila odourant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature* **452**: 1007–1011.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Data S1. Nucleotide and translated sequences of the endogenous *Or8*s in *Culex quinquefasciatus* (*CqOr113* and *CqOr118*) and the chimera *Or8*s (*CqOr113*+ and *CqOr118*−).