FUNCTIONAL CHARACTERIZATION OF INSECT CHEMORECEPTORS: RECEPTIVITY RANGE, EXPRESSION AND EVOLUTION

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FUNCTIONAL CHARACTERIZATION OF INSECT CHEMORECEPTORS: RECEPTIVITY RANGE, EXPRESSION AND EVOLUTION

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Functional characterization of insect chemoreceptors, following expression in heterologous cell systems, exemplifies the level of sophistication that insect chemoreception research has attained. "Abstract" (2011) by Nathaniel P. Wilkerson symbolizes the core event of heterologous gene expression in the fly: once the foreign genetic material has been injected into an embryo (see paper by Gonzalez et al. 2016), a developmental program, steered by an elegant ectopic gene transcription system, drives the expression of single olfactory receptor genes in target olfactory neurons in sensilla on the fly antenna. "Abstract" illustrates the tremendous power and the potential residing in a developing fly embryo, and again reminds us of the wonder of life.

Olfaction and taste are of critical importance to insects and other animals, since vital behaviours, including mate, food and host seeking, as well as predator and toxin avoidance, are guided by chemosensory cues. Mate and habitat choice are to a large extent determined by chemical signals, and chemoreceptors contribute accordingly to pre-mating isolation barriers and speciation. In addition to fundamental physiological, ecological and evolutionary consideration, the knowledge of insect taste and especially olfaction is also of great importance to human economies, since it facilitates a more informed approach to the management of insect pests of agricultural crops and forests, and insect vectors of disease.

Chemoreceptors, which bind to external chemical signals and then transform and send the sensory information to the brain, are at the core of the peripheral olfactory and gustatory system and have thus been the focus of recent research in chemical ecology. Specifically, emphasis has been placed on functional characterization of olfactory receptor genes, which are derived from three large gene families, namely the odorant receptors, gustatory receptors and ionotropic receptors. Spatial expression patterns of olfactory receptors in diverse chemosensory tissues provide information on divergent functions, with regards to ecologically relevant behaviours. On the other hand, characterization of olfactory receptor activation profiles, or "deorphanization", provides complimentary data on the molecular range of receptivity to the fundamental unit of the olfactory sense.

The aim of this Research Topic is to give an update on the breadth and depth of research currently in progress related to understanding the molecular mechanisms of insect chemoreception, with specific emphasis on the olfactory receptors.

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EDITORIAL
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Editorial: Functional Characterization of Insect Chemoreceptors: Receptivity Range and Expression

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The Editorial on the Research Topic

Functional Characterization of Insect Chemoreceptors: Receptivity Range, Expression, and Evolution

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Chemosensory systems play an oversize role in shaping the life of an insect, such that fundamental behaviors—mating, food choice and seeking, predator and parasitoid avoidance, and egg-laying—are strongly regulated by external chemical stimuli. The recent focus on the molecular mechanisms of chemosensory detection in insect chemical ecology research has identified canonical chemosensory receptors in insects that consist of odorant receptors (ORs), gustatory receptors (GRs), and ionotropic receptors (IRs). Much has been learned about the structure, function and evolution of chemosensory receptors since the initial discovery of ORs in *Drosophila melanogaster* in 1999, however, many outstanding questions remain. With this research topic, we aim to shine a light on expression patterns, reception properties, and evolutionary trends pertinent to insect chemosensory receptors. While intended to cover all chemosensory receptor families, this research topic is clearly biased toward ORs, reflecting the paucity of research conducted on GRs and IRs.

ECOLOGICAL AND BEHAVIORAL RELEVANCE OF CHEMORECEPTORS

The detection of ecologically relevant cues via chemoreceptors ultimately induces behavioral changes. The review by Depetris-Chauvin et al. provides an up-to-date look at chemical communication in flies and fitness-related behaviors, including courtship. The importance of both internal and external context for the interpretation of chemical cues is highlighted throughout the lifecycle of the fly. The authors suggest that plasticity in chemoreceptive behavior may be a result of chemoreceptor repertoire modulation, reflecting the distinct physiological requirements of various ecological environments inhabited at different life stages.

Modulation of chemosensory-based behaviors is a dynamic process that occurs subsequent to the processing of sensory stimuli, but the molecular mechanisms underlying such changes are not yet known. One supported hypothesis points toward a role for modulation of chemosensory gene expression in generating changes in behavior. Latorre-Estivalis et al. provide support for this

hypothesis, demonstrating the effects of blood-feeding and development on expression levels of OR and IR co-receptors in the important Chagas disease vector, *Rhodnius prolixus.*

EVOLUTION OF RECEPTOR TUNING PARADIGMS

Peripheral coding of signals contributes to the interpretation of chemosensory information in the insect nervous system. The hypothesis of peripheral combinatorial coding of chemical stimuli is contrasted to the labeled-line hypothesis. Re-examining these principles at the molecular level brings into play the concept of generalist vs. specialist receptors, with broader and more narrow receptor tuning ranges, respectively. Bohbot and Pitts explore these themes for insect ORs, and propose a general prevalence of specialized receptors, while acknowledging that pharmacological receptivity ranges of receptors may be broader. Exploring this concept further, Andersson et al. examine the principles of OR tuning in evolutionary contexts. Examples are provided for both broad and narrow tuning, and scenarios are presented wherein evolutionary conditions would favor tilting toward either model.

A central dogma concerning insect olfactory information flow is that one olfactory sensory neuron (OSN) expresses one OR subtype, and axons of OSNs expressing the same OR converge within the same glomerular cluster in the primary olfactory processing center of the brain, the antennal lobe. However, there are exceptions to these rules and Karner et al. showcase this, reporting the co-expression of four to six genomically clustered OR genes in the same OSN in a mosquito. These OSNs may thus serve as broadly tuned sensors.

Above and beyond investigations into model organisms with sequenced genomes, the advent of high-throughput transcriptomic sequencing (RNA-Seq) has led to a dramatic increase in the breadth of gene identification and characterization. Here, the application of RNA-Seq methodology is highlighted with a description of chemosensory gene families, including ORs and IRs, in the Colorado potato beetle (Liu et al.). The identification of beetle ORs with sexually biased expression patterns suggests a molecular basis for known sexually dimorphic olfactory-based behaviors.

A logical step following chemosensory receptor discovery is functional characterization. Receptor deorphanization is defined by the process of identifying key activating ligands for chemosensory receptors and describing their receptive range; a difficult task for non-model insects. Using the *in vivo* deorphanization systems that have been developed in *D. melanogaster* for transgenic expression and functional characterization of insect ORs, Gonzalez et al. provide detailed step-by-step protocols to facilitate widespread accessibility and adoption of this methodology.

Three reports in this Research Topic utilize fly transgenic systems to characterize ORs, (Gonzalez et al.) and pheromone receptors (PRs) (de Fouchier et al.; Bengtsson et al.) in moths, each making distinct contributions to the fundamental knowledge that underlies the molecular mechanisms of olfactory detection. Gonzalez et al. report that homologous ORs from two distantly related moth species respond similarly to the same set of odorant ligands. These data support functional conservation in homologous ORs and provide hypotheses concerning the interconnection of structure and function with respect to modeling odorant ligand interactions with critical amino acid residues in the receptor proteins.

While many hypotheses exist, there is still a prominent gap in the knowledge concerning the mechanism(s) underlying chemoreceptors' specific interaction with their chemical ligands. Almeida et al. provide an important contribution toward the theoretical framework of this knowledge with their examination of site-specific evolutionary rates in GRs and IRs in a non-insect lineage. Relaxed selective constraints are a prominent feature of duplicated genes, permitting neo-functionalization of redundant gene models. Furthermore, rapid evolution of specific amino acid residues is biased toward extracellular domains, which are predicted to be involved in ligand binding.

MOTH PHEROMONE RECEPTORS

The chemical ecology of moth pre-mating communication has been widely studied, from pheromone component identification and biosynthesis to PR characterization. This facet of insect chemical ecology has persisted in the spotlight largely due to the prominence of moths as agricultural pests, as well as the successfully demonstrated potential for hacking the olfactory system as a means of species specific biorational pest control. Accordingly, Zhang and Löfstedt provide a thorough review of state of the art knowledge on moth PRs with respect to sequence, function and evolution in the context of their pheromone ligands.

Exploring the underpinnings of moth mating systems, de Fouchier et al. report on two PRs that respond to similar, but not completely overlapping, sets of minor pheromone components. This report places these receptors in an evolutionary context, evaluating their position within broader lineages of moth PRs, as well as examining differential evolutionary pressures on specific amino acid residues. The latter point reiterates a need for a greater understanding of the mechanism(s) by which chemoreceptors interact with their ligands.

Continuing with the theme on evolution of PRs, Bengtsson et al. describe a codling moth OR that responds to a host plant volatile, pear ester, but clusters phylogenetically with the well-described sub-family clade of moth PRs. Its response to a host plant volatile was, at first glance, surprising, but the receptor displays hallmark features of PRs, namely, high specificity and sensitivity to its key ligand.

Evolution of pheromone communication requires the co-adaptation of pheromone and receptor, suggesting a degree of variation in the sequence and expression of each within a population. Alternative splicing represents one cellular mechanism whereby an increased diversity of protein products can stem from a relatively limited number of genes, providing functional plasticity in chemoreception. Here, Garczynski and Leal provide the first known report on splicing of the 3'/C-terminal region of PR sequence. The functional implications

of this remain unknown, and further research is required on structure function relationships and ligand binding properties of alternatively spliced receptors.

CONCLUDING REMARKS

We are very grateful to all authors who contributed articles to this Topic, illustrating most of the facets of studies currently conducted on insect chemoreceptors. We also thank all reviewers and affiliated scientific editors who helped us in reaching the highest quality standards, as well as the Frontiers editorial team for invaluable and consistent support and encouragement.

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REVIEW
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Chemicals and chemoreceptors: ecologically relevant signals driving behavior in *Drosophila*

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Insects encounter a vast repertoire of chemicals in their natural environment, which can signal positive stimuli like the presence of a food source, a potential mate, or a suitable oviposition site as well as negative stimuli such as competitors, predators, or toxic substances reflecting danger. The presence of specialized chemoreceptors like taste and olfactory receptors allows animals to detect chemicals at short and long distances and accordingly, trigger proper behaviors toward these stimuli. Since the first description of olfactory and taste receptors in *Drosophila melanogaster* 15 years ago, our knowledge on the identity, properties, and function of specific chemoreceptors has increased exponentially. In the last years, multidisciplinary approaches combining genetic tools with electrophysiological techniques, behavioral recording, evolutionary analysis, and chemical ecology studies are shedding light on our understanding on the ecological relevance of specific chemoreceptors for the survival of *Drosophila* in their natural environment. In this review we discuss the current knowledge on chemoreceptors of both the olfactory and taste systems of the fruitfly. We focus on the relevance of particular receptors for the detection of ecologically relevant cues such as pheromones, food sources, and toxic compounds, and we comment on the behavioral changes that the detection of these chemicals induce in the fly. In particular, we give an updated outlook of the chemical communication displayed during one of the most important behaviors for fly survival, the courtship behavior. Finally, the ecological relevance of specific chemicals can vary depending on the niche occupied by the individual. In that regard, in this review we also highlight the contrast between adult and larval systems and we propose that these differences could reflect distinctive requirements depending on the change of ecological niche occupied by *Drosophila* along its life cycle.

Keywords: Olfaction, taste, receptor, *Drosophila*, attraction, repulsion, ecological niche

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Introduction

Chemoreception is defined as the physiological response to a chemical stimulus. Depending on the spatial scale, a classical division exists between olfaction and taste chemoreception. Olfaction is involved in the detection of volatile molecules coming from long distances, while taste is a contact sense that allows detection of molecules at a short distance. Highly volatile hydrophobic molecules can be rapidly transported by air and, once they reach the

living organism, activate olfactory receptors. On the contrary, hydrophilic molecules are less volatile and they most likely activate taste receptors when presented at a short distance. This definition might not be suitable for aquatic environments where solubility instead of volatility is the determinant factor for long-distance transport of molecules (Mollo et al., 2014).

One of the favorite model organisms for the study of olfaction and taste perception is the fly *Drosophila melanogaster*. In the last two decades, and due to its amazing repertoire of genetic tools, *Drosophila* has been at the leading front in the discovery of chemoreceptors and chemoreceptive neuronal pathways that account for the behavioral responses toward ecologically relevant chemicals. Even more, the extensive work done in *Drosophila* helps us to better understand the chemoreceptive systems of insects relevant for human's health, such as the mosquitos *Anopheles gambiae* and *Aedes aegypti,* dangerously efficient vectors of malaria and Dengue hemorrhagic fever.

Flies are able to perceive relevant chemical cues present in their food, in host plant, and those produced by conspecific. Attractive odors and tastants in the food can induce feeding, while toxic compounds present in food or produced by host plants trigger avoidance. Before activating the oviposition motor program, female flies carefully analyze the chemical composition of the substrate. Also, conspecific chemical cues are essential for aggregation, aggression, and courtship. All of these effects depend on proper detection of chemical cues at the level of olfactory and gustatory receptors present in dedicated structures.

Here we will review the extensive recent research focused on detection of ecologically relevant chemicals in flies and its behavioral consequences. Firstly, we will very briefly outline the olfactory and gustatory system of fly adults and larvae, giving more emphasis to the description of the different families of chemoreceptors. Secondly, we will present several examples of chemoreceptors involved in the detection of chemical signals that impact on behaviors relevant for fly survival, such as feeding, toxic compounds avoidance, and oviposition site and sexual partner selection. Finally, we will review and discuss the ecological relevance of specific chemicals and chemoreceptors in the context of the particular requirements of two stages of *Drosophila* life cycle, the larva and the adult fly.

Olfactory and Gustatory Chemoreceptors in Flies: Several Receptors Distributed in Several Families

The olfactory organs of the adult fly are located on the third antennal segment (also known as funiculus) and on the maxillary palps, where three different types of sensilla, the basiconic, the trichoid, and the coeloconic, harbor the olfactory sensory neurons (OSNs) (**Figure 1A**). In the OSNs, olfactory receptors directly contact their specific ligands. From the periphery, OSNs send axonal projections to specific glomeruli in the antennal lobe, the first olfactory relay center in the brain. In the antennal lobe, the odor signals are processed by local interneurons and projection neurons. Local interneurons connect different glomeruli mainly triggering later inhibition (Silbering and Galizia, 2007), and projection neurons transmit the olfactory trace to higher centers in the lateral horn and mushroom bodies (reviewed in Stocker, 1994; Laissue and Vosshall, 2008). Careful anatomical description of the olfactory system allowed building a near complete map of OSN's connectivity. OSNs expressing the same olfactory receptor project into the same unique glomerulus in the antennal lobe (Couto et al., 2005; Fishilevich and Vosshall, 2005; Goldman et al., 2005). In addition, OSNs harbored in different type of sensilla project into distinct regions of the antennal lobe, highlighting the level of topographic organization of the olfactory system (Couto et al., 2005).

In contraposition to olfactory organs, taste organs are widely distributed in the adult body, with external gustatory centers on the proboscis's labellum, legs, wings, and female genitalia, and internal taste structures in the pharynx (**Figure 1A**). The labellum is the principal taste organ of the adult fly and it harbors two major types of sensilla, the taste bristles and taste pegs, wherein gustatory receptors expressed in gustatory receptor neurons (GRNs) directly detect tastant. In the pharynx, the labral sense organ (LSO), the ventral and dorsal cibarial sense organs (VCSO and DCSO), and a ventral and a dorsal row of "fish-trap" bristles allow taste detection after ingestion (reviewed in Stocker, 1994; Montell, 2009). From the taste organs located in the mouth parts, proboscis, and legs, GRNs transmit directly or through activation of interneurons the gustatory information to the subesophageal ganglion (SOG), a dedicated taste center in the brain (Wang et al., 2004). Some taste-like sensilla are also present on the genitalia and on the wing margin, but their precise role is still under investigation (Boll and Noll, 2002; Yanagawa et al., 2014). In the SOG, axonal projections coming from different peripheral tissues are segregated even if they contain the same receptor (Wang et al., 2004). Even more, bitter and sugar sensing neurons clearly segregate in the SOG, demonstrating that the first gustatory relay center displays a topographic and functional organization (Thorne et al., 2004; Wang et al., 2004).

The external chemosensory organs of the larvae are all located in the cephalic lobe with the exception of some putative taste organs in thoracic and abdominal segments (Dambly-chaudière and Ghysen, 1986; Scott et al., 2001) (**Figure 1B**). Larval olfactory structures are located in the dorsal organ, while external gustatory structures are mainly distributed between the terminal and ventral organs, and to a lesser extent, the dorsal organ. In addition, three internal pharyngeal organs, the dorsal, ventral, and posterior pharyngeal sense organs (DPS, VPS, and PPS, respectively) include mainly taste sensilla (Stocker, 2008). Similar to the case of the adult gustatory system, the larval SOG shows a certain topographic and functional organization although in the larvae there is no complete segregation between external and internal GRNs axonal projections (Colomb et al., 2007; Kwon et al., 2011). The dorsal organ is composed of the central "dome" that harbors the dendrites of the 21 larval OSNs and a few putative taste sensilla (Gerber and Stocker, 2007; Stocker, 2008). This small number of OSNs contrasts with the around 1300 OSNs that are present in adults. Despite these numeric differences, the adult and larval olfactory pathways share the same design (Stocker, 2009). Nonetheless, the larval olfactory system is not just a reduced version of the adult system because some olfactory receptors are only

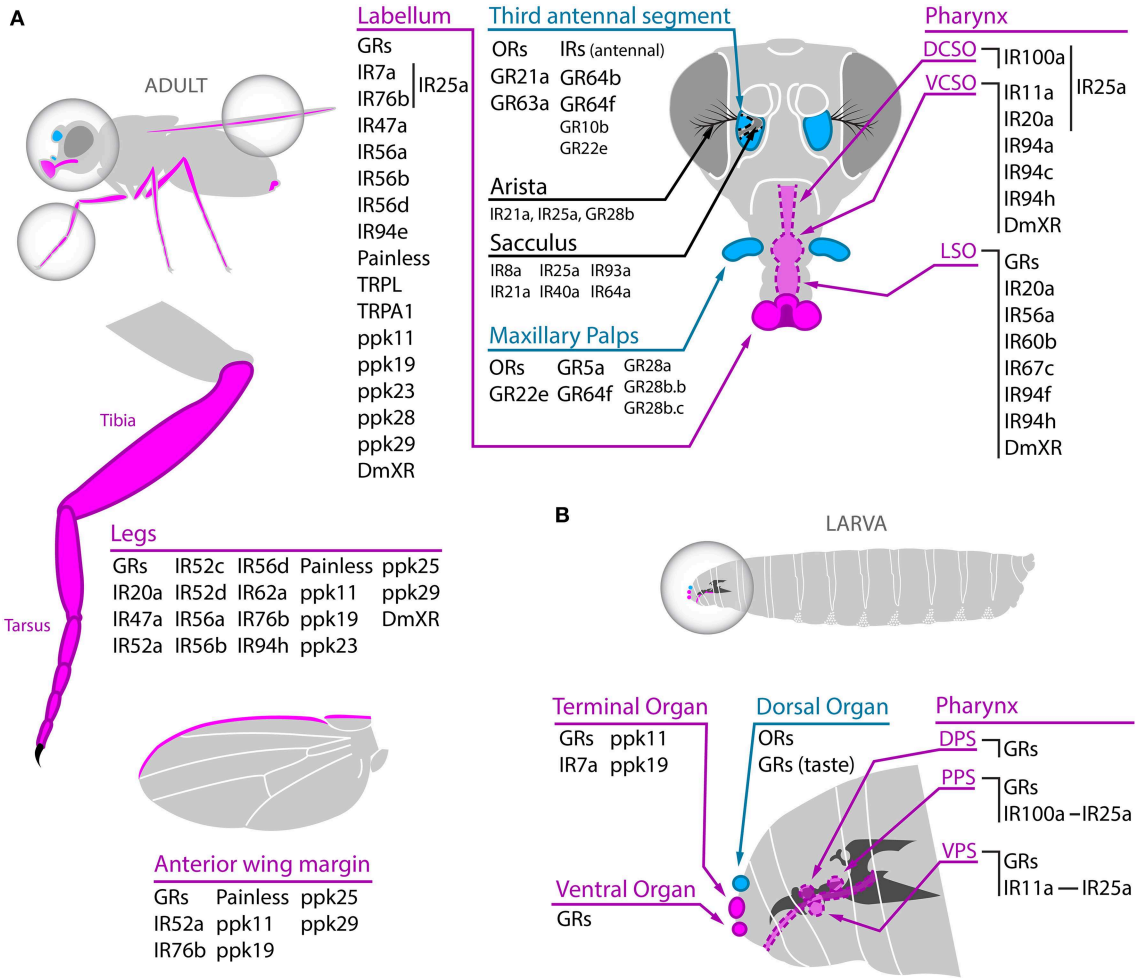

FIGURE 1 | Chemoreceptors expressed in adult (A) and larval (B) olfactory and gustatory organs. (A) Adult taste organs (magenta) are located on the proboscis's labellum, the tarsus and tibia of the leg, the anterior wing margin, the female genitalia (not shown), and in internal taste structures in the pharynx (DCSO, VCSO, and LSO). Within those taste organs, GRs, several members of the IR family, some members of the TRP family (Painless, TRPL and TRPA1), ppk channels (ppk11, ppk19, ppk23, ppk25, ppk28, and ppk29), and the insect orphan receptor DmXR work as chemoreceptors (although some of them have not been confirmed as *bona fide* receptors yet). At least IR7a, IR76b in the labellum and IR11a, IR20a, and IR100a in internal taste organs are coexpressed with IR25a. The third antennal segment and the maxillary palps are the adult olfactory organs (cyan) and they mainly harbor ORs and IRs, although expression of 7 GRs (GR10b, GR22e, the CO_2 receptors GR21a and GR63a, and the sugar receptors GR5a, GR64b, and GR64f) has also been detected. Plus, GR28a, GR28b.b, and GR28b.c show expression in maxillary palp neurons of unknown function. IRs and GRs expressed in the arista and the sacculus and with unknown function are also displayed in the antenna scheme. **(B)** In the larva taste receptors, mainly GRs and some IRs, are localized in the terminal, ventral, and to a lesser extent dorsal organs as well as in several internal taste organs (DPS, PPS, and VPS). As in adults, IR11a and IR100a are expressed in gustatory receptor neurons coexpressing IR25a. The dorsal organ harbors ORs involved in olfactory responses. Dotted line indicates internal or deeper structures.

expressed in the larval stage (Stocker, 2008). Even more, the larval local interneurons in the antennal lobe do not keep resemblance with their adult counterparts and in the larva they tightly connect gustatory and olfactory centers (Thum et al., 2011).

Regarding the chemoreceptors, in flies more than 150 receptors are distributed in three principal families, the gustatory receptors (GRs), the odorant receptors (ORs), and the ionotropic receptors (IRs). In addition, some members of the TRP family and degenerin/epithelial sodium channel/pickpocket (ppk) channels as well as the insect orphan G-protein-coupled DmXR are either *bona fide* chemoreceptors or they are tightly involved in chemoreception in flies (**Table 1**).

In mammals, most chemoreceptors are classic seven-transmembrane G-protein-coupled receptors (GPCRs) (Chandrashekar et al., 2006; Spehr and Munger, 2009). In insects, GRs and ORs are also seven transmembrane domain proteins with a no amino acid sequence homology compared to mammalian ORs and GRs (Vosshall et al., 1999; Clyne, 2000; Scott et al., 2001); however, insect ORs have a topology opposite to mammalian GPCRs, with cytoplasmic N-termini and extracellular C-termini (Benton et al., 2006). A phylogenetic analysis indicated that insect OR family is an expanded lineage within the ancestral insect GR family (Robertson et al., 2003), highlighting a common evolutionary origin. In addition, insect IRs

TABLE 1 | Families of chemoreceptors in *Drosophila melanogaster*.

Family	Modality	Localization	Coreceptor	References
ORs (60 genes)	Olfaction	Adult antenna (BS and TS) and maxillary palps Larval dorsal organ	OR83b (ORCO)	Kurtovic et al., 2007; Van der Goes van Naters and Carlson, 2007; Laissue and Vosshall, 2008; Stocker, 2008
	Sexual pheromones detection	Adult antenna		
IRs (61 genes)	Olfaction	Adult antenna (CS)	IR8a, IR25a, and IR76b	Benton et al., 2009; Ai et al., 2010, 2013; Croset et al., 2010; Abuin et al., 2011; Grosjean et al., 2011; Zhang et al., 2013a; Koh et al., 2014
	Taste	Adult labellum, internal taste organ, legs, and wing margin		
		Larval terminal organ and internal taste organs		
	Sexual pheromones detection? (IR56c and IR56d)	Adult legs		
	Unknown function	Arista and sacculus of the adult antenna		
GRs (68 genes)	Olfaction (GR21a and GR63a. GR5a?, GR10b?, GR22e?, GR64b?, and GR64f?)	Adult antenna and/or maxillary palps	GR21a-GR63a heterodimer?	Clyne, 2000; Dunipace et al., 2001; Scott et al., 2001; Bray and Amrein, 2003; Colomb et al., 2007; Jones et al., 2007; Kwon et al., 2007, 2011; Miyamoto and Amrein, 2008; Thorne and Amrein, 2008; Montell, 2009; Lee et al., 2010; Park and Kwon, 2011; Liman et al., 2014; Ling et al., 2014; Fujii et al., 2015
	Taste	Adult labellum, internal taste organs, legs, and wing margin	Heteromultimers with GR5a (sweet) and GR66a (bitter)	
		Larval terminal, dorsal and ventral organs, and internal taste organs		
	Sexual pheromones detection? (GR32a, GR33a, and GR68a)	Adult legs	GR32a-GR33a heterodimer?	
	Hygro/thermoreception	Adult arista		
	Peripheral propioperception	Adult leg		
	Unknown function	Intestine, Johnston's organ, abdominal multidendritic neurons, central nervous system		
TRP channels	Taste (TRPA1, TRPL, and Painless)	Adult labellum, wing margin, and legs		Al-Anzi et al., 2006; Kim et al., 2010; Neely et al., 2011; Fowler and Montell, 2013; Zhang et al., 2013b
	Phototaxis, thermotaxis, hygrosensation, gravitotaxis, and propioception	Optic structures, Johnston's organ, multidendritic neurons, mechanosensory bristles, and femoral chordotonal neurons		
ppk channels	Taste (ppk11, ppk19, and ppk28)	Adult labellum, wing margin, and legs	ppk11-ppk19 heterodimer?	Liu et al., 2003a,b, 2012; Lu et al., 2012; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; Zelle et al., 2013; Guo et al., 2014; Mast et al., 2014; Vijayan et al., 2014
		Larval terminal organ		

(Continued)

TABLE 1 | Continued

Family	Modality	Localization	Coreceptor	References
	Sexual pheromones detection? (ppk23, ppk25, and ppk29)	Adult legs	ppk23-ppk29 heterodimer? ppk23-ppk25-ppk29 complex?	
	Aggregation pheromones detection? (ppk23 and ppk29)	Larval terminal organ and one internal taste cell		
	Mechanoperception	Class IV dendritic arborization neurons		
	Liquid clearance?	Trachea		
DmXR	Taste?	Adult labellum, legs, and internal taste organs		Mitri et al., 2004, 2009

BS, TS, and CS stand for basiconic, trichoid, and coeloconic sensilla, respectively. Many members of these families have not been confirmed as bona fide receptors yet (see main text for details).

are ligand-gated ion channels involved in chemoreception and they belong to the superfamily of ionotropic glutamate receptors (Benton et al., 2009). Interestingly, a subgroup of IRs are expressed in the antenna and, contrary to what is seen in most of the ORs, they are highly conserved within insects, both in sequence and expression pattern, suggesting that antennal IRs might represent the ancestral olfactory receptor family in insects (Croset et al., 2010). Below, we will describe in more detail some characteristics of these families of chemoreceptors, separating between those involved in olfaction and the ones dedicated to taste perception.

Chemoreceptors and Olfactory Detection

The first family of chemoreceptors described in *Drosophila* was that of odorant receptors (ORs) comprising 60 genes expressed in subpopulations of OSNs (Clyne et al., 1999; Vosshall et al., 1999) mainly in basiconic and trichoid sensilla. The 60 OR genes give rise through alternative splicing to 62 proteins, and of those expressed in the adult system some are exclusively expressed in the antenna, and some others in the maxillary palps (Laissue and Vosshall, 2008). In addition, 13 ORs are only detectable in olfactory organs of the larvae (Couto et al., 2005; Fishilevich et al., 2005; Kreher et al., 2005) (**Figure 1** and **Table 1**). OR83b (also known as ORCO) is expressed in all adult maxillary palp OSNs, around 75% of the antennal OSNs, and in all the larval OSNs. ORCO forms heterodimeric complexes with other OR protein (Larsson et al., 2004; Neuhaus et al., 2005) and, with a few exceptions, only one pair "ORCO-conventional OR" is expressed per OSN (Couto et al., 2005). Furthermore, ORCO's expression is necessary both in adults and in larvae for electrophysiological and behavioral responses to several odorants, demonstrating that ORCO is an essential coreceptor for all the ORs (Larsson et al., 2004).

By using a mutant antennal neuron that lacks its endogenous chemoreceptors (the "empty neuron" system; Dobritsa et al., 2003), the group of Carlson performed extensive electrophysiological characterizations of ORs responsiveness toward relevant food-derived odorants, both in the adult antenna and the larval olfactory system (Hallem et al., 2004; Hallem and Carlson, 2006; Kreher et al., 2008; Mathew et al., 2013). Individual receptors range along a continuum from narrowly to broadly tuned although, in general, reducing odor concentration reduces the number of ORs activated (Hallem and Carlson, 2006). In contraposition to the extensive analysis of electrophysiological responses at the periphery, little is known about the relevance of specific ORs in driving behavior. In larvae, some odors weakly activate ORs but trigger strong behavioral responses and, on the contrary, other odors can strongly activate ORs but elicit weak behavioral responses (Mathew et al., 2013; Grewal et al., 2014); this highlights that odor coding in higher-olfactory centers is a relevant process that modulates odor-trigger behaviors.

Drosophila olfactory responses also rely on the activity of IRs (Benton et al., 2009). Of the 61 members of the IR family, 18 are normally expressed in the adult antenna in coeloconic sensilla, the sacculus, or the arista, while no expression has been described so far in olfactory organs of the larvae (Benton et al., 2009; Croset

et al., 2010) (**Figure 1** and **Table 1**). In the adult olfactory system, IRs act in combination of up to three subunits with IR8a or IR25a and maybe other IRs serving as general coreceptors for odor-specific IRs (Abuin et al., 2011). A comparative electrophysiological analysis of the two olfactory subsystems, ORs and IRs, in the adult antenna revealed some differences in ligand specificity. In general, IR8a$^+$ OSNs respond to carboxylic acids and some aldehydes, whereas IR25$^+$ OSNs are preferentially activated by amines, and OR$^+$ OSNs are more dedicated to the detection of esters, alcohols, and ketones (Silbering et al., 2011).

In addition to ORs and IRs, pioneer expression analysis indicated that four gustatory receptors, GR21a, GR63a, GR10b, and GR22e, are expressed in the adult antenna (Dunipace et al., 2001; Scott et al., 2001), and at least GR21a and GR63a are *bona fide* olfactory receptors (Jones et al., 2007; Kwon et al., 2007). Plus, a very recent study demonstrated the expression of three sugar GRs, GR5a, GR64a, and GR64f, in adult olfactory organs although their function in these cells has not been determined yet (Fujii et al., 2015) (**Figure 1** and **Table 1**).

Chemoreceptors and Gustatory Detection

At least three families of chemoreceptors or channels are involved in gustatory responses: GRs, IRs, and TRPs channels. Of those, GRs were the first to be characterized as contact receptors and, up to now, the majority of taste and pheromones receptors found in flies belong to this extensive family of 68 members expressed differentially in all the taste organs of the adult and the larva (Clyne, 2000; Dunipace et al., 2001; Scott et al., 2001). At least 38 GRs are expressed in the labellum (Weiss et al., 2011) and 28 GRs in the leg (Ling et al., 2014). A minimum of 39 GRs is present in larval taste organs, and most of them are presumed to be bitter receptors (Colomb et al., 2007; Kwon et al., 2011) (**Figure 1** and **Table 1**). In the labellum, two GRs, GR5a and GR66a, are extensively expressed in non-overlapping GRNs populations. GR5a$^+$ neurons respond to sugar and elicit feeding behavior, while bitter compounds activate GR66a$^+$ neurons and trigger avoidance behavior (Dahanukar et al., 2001; Chyb et al., 2003; Thorne et al., 2004; Marella et al., 2006).

In contrast to the simple heterodimers of ORs and antennal IRs, GRs seem to act as heteromultimeric complexes. Eight GRs, among them GR5a, are expressed in a combinatorial manner giving rise to a minimum of eight sets of sweet-sensing neurons in adult taste organs (Fujii et al., 2015). GR66a, GR93a, and GR33a appear to be coexpressed in most if not all bitter-sensing GRNs in the labellum (Lee et al., 2009; Moon et al., 2009); plus, GR33a is necessary in those GRNs for the response to all bitter compounds tested (Moon et al., 2009). Moreover, in larvae GRs are expressed combinatorially and up to 17 subunits could be present in a single GRN (Kwon et al., 2011).

In addition to GRs, evidence from expression profile analysis and loss of function studies point to other proteins as taste receptors or at least as essential components for certain taste modalities. Several IR members are expressed in taste organs where they could act as taste receptors. Both in adults and in larvae, IR25a is coexpressed with IR7a, IR11a, and IR100a in taste organs (Croset et al., 2010). In adults, IR76b is located in L-type sensilla in the labellum where it acts as a low-salt detector, and additional expression is also seen in GRNs in the leg tarsi and wing margins (Zhang et al., 2013a). Very recently, several members of the non-antennal IRs were found in almost all the taste organs of the adult fly (Koh et al., 2014) (**Figure 1** and **Table 1**). In addition, at least three members of the TRP channels, TRP1, Painless, and TRPL are expressed in bitter neurons in the labellum where they are involved in detection of aversive compounds (Al-Anzi et al., 2006; Kim et al., 2010; Zhang et al., 2013b). Also, several ppk channels are expressed in taste neurons where they are required for relevant taste modalities such as low-salt detection (Liu et al., 2003b) and intraspecific chemical communication in larvae (Mast et al., 2014), and water perception (Cameron et al., 2010) and chemical communication during courtship in adults (Liu et al., 2012; Lu et al., 2012; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014). Finally, DmXR, a receptor homologous to metabotropic glutamate receptors that has lost the ability to bind glutamate (Mitri et al., 2004), may also act as a taste receptor. It is expressed in GRNs in the labellum, the leg, and internal taste organs (LSO and VCSO) and it was originally described as a L-canavanine amino acid receptor, even if its exact role on chemoreception is still under debate (Mitri et al., 2009; Lee et al., 2012) (**Figure 1** and **Table 1**).

Ecological Relevance of Specific Chemoreceptors

Genomic comparative studies highlight the rapid evolution of chemoreceptors both in number and identity (Robertson et al., 2003; Croset et al., 2010). This feature led to the hypothesis that changes at the level of chemosensory systems contribute to the diversification of behaviors (Cande et al., 2013). Evidence in favor of this hypothesis comes mainly from comparative studies of closely related *Drosophila* species with different behaviors, as it is the case of *D. melanogaster* and *D. sechellia*. *D. melanogaster* is a generalist species that can survive in several fruit substrates, while *D. sechellia* is a host-plant specialist. Interestingly, *D. melanogaster* has a complex olfactory system that allows detection of hundreds of fruit-derived odors; *D. sechellia*, on the contrary, has lost many chemoreceptors that are not relevant for its very specialized ecology (Stensmyr et al., 2003; Cande et al., 2013). Although a very provocative hypothesis, it is difficult to prove that mutations in chemoreceptor gene *loci* are important driving forces behind behavioral change. Nonetheless, there is no doubt that the current chemoreceptors allow detection of ecologically relevant chemicals present in the fruitfly's environment. In this section we will discuss the ecological relevance of specific chemoreceptors related to behaviors such as food searching and the analysis of its composition, avoidance of toxic or bitter compounds, oviposition site selection, and the search for a sexual partner (**Table 2**).

Chemoreceptors Involved in Food Sources Searching and Food Composition Analysis

During larval stage, *Drosophila* individuals increase their size in about 200 times in 4 days. Such high growth rate requires an immense amount of energy, and to obtain it larvae have to

TABLE 2 | Ligands and chemoreceptors ecologically relevant in *Drosophila* adult flies.

Ligand		Receptor/Putative receptor		Evoqued behavior	References
		Identity	**Localization**		
BASIC MOLECULES/IONS					
CO_2	In air	GR21a and GR63a	Antenna	Avoidance	Jones et al., 2007; Kwon et al., 2007
	In water	Unknown taste receptor	Labellum	Attraction	Fischler et al., 2007
H^+		IR64a-IR8a	Antenna	Avoidance	Ai et al., 2010, 2013
Salt	Low concentrations	IR76b in adults ppk11 and ppk19 in larvae	Labellum	Attraction	Balakrishnan and Rodrigues, 1991; Liu et al., 2003a,b; Zhang et al., 2013a
	High concentrations	Unknown receptor (ppk11 and ppk19?)	Labellum	Avoidance	Balakrishnan and Rodrigues, 1991; Liu et al., 2003a,b; Zhang et al., 2013a; Alves et al., 2014
Water		ppk28	Labellum	Drinking behavior	Cameron et al., 2010
FOOD-DERIVED CHEMICALS					
Acetic acid		IRs and ORs?	Antenna	Avoidance	Joseph et al., 2009; Abuin et al., 2011; Silbering et al., 2011; Ai et al., 2013
		Unknown taste receptor		Stimulate oviposition	Joseph et al., 2009
Aldehydes		IR8a + other IRs	Antenna		Silbering et al., 2011
Amines		IR92 + IR25a + other IRs	antenna	Attraction	Silbering et al., 2011; Min et al., 2013
Ammonia		IR92 + other IRs	Antenna	Attraction	Min et al., 2013
Carboxylic acids		IRs and ORs?	Antenna	Avoidance	Hallem and Carlson, 2006; Abuin et al., 2011; Silbering et al., 2011
		Unknown receptor	Bitter and sweet neurons	Avoidance and modulation of bitter/sugar mix perception	Charlu et al., 2013; Chen and Amrein, 2014
Esters, alcohols, and ketones		Mainly ORs	Antenna		Silbering et al., 2011
Ethylphenols	(Derived from dietary antioxidants)	OR71a in adults OR94a in larvae	Maxillary palps and larval terminal organ	Attraction and stimulation of feeding and oviposition	Dweck et al., 2015
Farnesol		OR83c	Antenna	Attraction	Ronderos et al., 2014
Fatty acids		Unknown receptor	Sweet neurons		Masek and Keene, 2013
Glycerol		GR64e		Proboscis extension and feeding preference	Wisotsky et al., 2011
Limonene		OR19a	Antenna	Stimulate oviposition	Dweck et al., 2013

(Continued)

TABLE 2 | Continued

Ligand		Receptor/Putative receptor		Evoqued behavior	References
		Identity	**Localization**		
PAA and PA		IR84a	Antenna	Aphrodisiac for males	Grosjean et al., 2011
Sugars	Fructose	GR43a	Labellum, legs, and internal taste organs	Attraction	Miyamoto et al., 2012
	Glucose, maltose, sucrose, trehalose	GR5a, GR61a, and GR64a-f *	Labellum, legs, and internal taste organs	Attraction	Chyb et al., 2003; Thorne et al., 2004; Wang et al., 2004; Dahanukar et al., 2007; Jiao et al., 2007, 2008; Slone et al., 2007; Fujii et al., 2015
BITTER OR TOXIC COMPOUNDS					
Caffeine		GR66a, GR33a, and GR93a	Labellum and legs	Avoidance	Moon et al., 2006, 2009; Lee et al., 2009
Camphor		TRPL	Labellum	Avoidance	Zhang et al., 2013b
Citronellal		Unknown OR + TRPA1 §	Antenna	Avoidance	Kwon et al., 2010
DEET		GR32a, GR33a, and GR66a	Labellum	Antifeedant effect	Lee et al., 2010
		Unknown ORs	Antenna	Avoidance and inhibition of food odor perception	Ditzen et al., 2008; Das et al., 2014
Geosmin		OR56a	Antenna	Oviposition and feeding avoidance, negative taxis, and decrease attraction toward food odors	Stensmyr et al., 2012
L-canavanine		GR8 and GR66a	Labellum	Avoidance	Lee et al., 2012
Wasabi (isothiocyanate)		Painless (TRP channel)	Labellum, legs, marginal wing, and internal taste organs	Avoidance	Al-Anzi et al., 2006
CONSPECIFIC SIGNALS					
7-T &	Male hydrocarbon	GR32a-GR33a	Leg	Anti-aphrodisiac for intra and interspecific males	Miyamoto and Amrein, 2008; Moon et al., 2009; Fan et al., 2013
		ppk23-ppk29	Leg	Anti-aphrodisiac for intraspecific males	Thistle et al., 2012
7,11 HD and 7,11 ND °	Female hydrocarbons	GR68a, GR39a	Male leg	Aphrodisiac for intraspecific males	Bray and Amrein, 2003; Watanabe et al., 2011
		ppk23-ppk25-ppk29	Leg	Aphrodisiac for intraspecific males, female receptivity promotion	Liu et al., 2012; Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014
		IR52c-IR52d	Leg	Aphrodisiac for intraspecific males	Koh et al., 2014
cVA +	Male pheromone	OR67d and OR65a	Antenna	- Anti-aphrodisiac for males, female receptivity promotion, and aggression promotion (OR67d-dependant). - Aggression reduction after long-term exposure (OR65a-dependant)	Ejima et al., 2007; Kurtovic et al., 2007; Van der Goes van Naters and Carlson, 2007; Wang and Anderson, 2010; Liu et al., 2011
		Ppk23-ppk29	Leg	Anti-aphrodisiac for males	Thistle et al., 2012

(Continued)

TABLE 2 | Continued

Ligand		Receptor/Putative receptor		Evoqued behavior	References
		Identity	Localization		
CH503	Male hydrocarbon	Unknown receptor		Anti-aphrodisiac for males	Yew et al., 2009; Ng et al., 2014
Fly odors (male and female)		OR47b and OR88a	Antenna	Aphrodisiac for males	Van der Goes van Naters and Carlson, 2007; Wang et al., 2011
(Z)-5 and 7-tetradecenoic acid	Larval fatty acids	ppk23-ppk29	Larval terminal organ	Larval aggregation	Mast et al., 2014

PAA is phenylacetic acid and PA, phenylacetaldehyde. Some of the proteins listed here have not been confirmed as bona fide receptors yet (see main text for details).
#In larvae, low-salt solutions seem to be detected by ppk11 and ppk19 (Liu et al., 2003b).
**In larvae, GR43a is the only sugar receptor (Mishra et al., 2013).*
® In Anopheles gambae mosquitoes the TRPA1 ortholog responds directly to citronellal (Kwon et al., 2010).
& 7-T also promotes male-male aggression by acting in the same neuronal pathway as cVA (Wang et al., 2011).
◦ 7,11 HD produced by females inhibits courtship from males of other Drosophila species acting through undescribed receptors (Billeter et al., 2009).
+ In addition to the phenotypes in courtship and aggression, cVA was described as an aggregation factor for males and females (Bartelt et al., 1985).

eat constantly (Tennessen and Thummel, 2011). The group of Vosshall studied the relevance of general odor detection for survival during this critical period when larvae are foraging for food. In a situation of excess food, anosmic foraging larvae show a survival rate comparable to that of larvae with an intact olfactory system. However, under limited food conditions or high competition, larvae need their sense of olfaction to localize a new food source (Asahina et al., 2008). Thus, the evolutionary advantage of an olfactory system tuned to food odors is reasonably evident. The importance of olfaction detection is also evident under mixed-age high-density laboratory cultures when younger larvae could turn toward cannibalism. In that scenario, chemosensory cues released from victim's injuries during the first attack could be relevant to induce aggregation and further collective cannibalistic behavior (Vijendravarma et al., 2013).

Larvae show general attraction toward a big range of odors of varied chemical characteristics, such as acids, alcohols, ketones, aldehydes, esters, and to a lesser extent, some terpenes and aromatics (Fishilevich et al., 2005; Khurana and Siddiqi, 2013). Among these odorants, some are present in common tropical fruits where *Drosophila* flies are naturally found (Khurana and Siddiqi, 2013). Interestingly, these odorants elicit stronger attractive responses than odors produced by non-fruit substrates, including flowers, leaves, and bark (Khurana and Siddiqi, 2013). However, not only the chemical identity of the odorant but also its concentration constitutes relevant information coded by the olfactory system. Depending on the concentration, some odorants could trigger responses that range from indifference to attraction or in some cases, even repulsion (Stensmyr, 2003). The dose-responses curves could be different even for odorants with related chemical structure, so each odorant should be analyzed individually (Khurana and Siddiqi, 2013). On the other hand, in the taste system, the concentration of the tastant is also a relevant cue. For example, both larvae and adult flies prefer low and reject high concentration of salts (Miyakawa, 1981; Zhang et al., 2013a). In this sense, the concentration of the chemical must be taking into account when analyzing the effects on the olfaction and taste systems.

In adult flies, food-derived odors also trigger attraction. At long distances, the presence of vinegar, or even acetic acid alone, is sufficient to trigger upwind flight attraction in starved flies (Becher et al., 2010; Lebreton et al., 2012). At short distances, fly odors together with food odors elicit attraction (Ruebenbauer et al., 2008; Lebreton et al., 2012). Some food-derived odors activate several olfactory receptors while others target only few or just one receptor (Hallem and Carlson, 2006). For example, OR83c receptor is essential for the detection of farnesol, a compound found in citrus fruit peel that triggers attraction in adult flies (Ronderos et al., 2014). A very recent study demonstrated that flies are attracted to antioxidants supplemented food thanks to their detection through olfactory cues. Polyphenol antioxidants normally present in fly food are converted by yeast into ethylphenols, and these strongly activate OR71a in adults and OR94a in larvae, leading to attraction in both stages and promoting feeding and oviposition in adults (Dweck et al., 2015). Dietary antioxidants offer protection against oxidative stress in flies (Jimenez-Del-Rio et al., 2010), so an olfactory pathway dedicated to the

detection of antioxidant-supplemented food may, most likely, increase *D. melanogaster* fitness. In addition, IR92$^+$ neurons detect ammonia and several different amines and activate a specific neuronal pathway dedicated to attractive behavior (Min et al., 2013). Interestingly, ammonia and amines are highly attractive for both flies and mosquito, although the ecological context in which they find them is different; flies may perceive ammonia and amines produced by fruit decomposition, while mosquito are attracted to the same compounds but emanated from animal hosts (Meijerink et al., 2001; Min et al., 2013). Anyway, in both species, a specific receptor to ammonia and amines appears to be important for the detection of a food source.

Another interesting case of chemoreception of ecologically relevant signals is that of CO_2 detection. CO_2 is a complex signal for the fly since it is a component of the aversive *Drosophila* stress odorant (Suh et al., 2004) and also an indicator of food source suitability (Faucher et al., 2006). It is sensed through GR21a and GR63a in the olfactory system and mediates avoidance behavior both in adult and in larvae (Jones et al., 2007; Kwon et al., 2007). This aversive olfactory effect is also mediated by IR64a via the solubilization of CO_2 in the antennal hemolymph leading to the production of H^+ (Ai et al., 2010). The aversive response of atmospheric CO_2 depends on life stage, sex, and olfactory context (Faucher et al., 2006). Furthermore, adult flies also perceive CO_2 in solution (carbonated water) through unknown taste receptors, and the taste of carbonated water mediates acceptance behavior (Fischler et al., 2007). Direct orthologs of GR21a and GR63a are present in mosquitos like *A. aegypti* and *A. gambiae*, and these are also dedicated to CO_2 perception. However, the underlying neuronal circuits do not seem to be conserved because in *Drosophila* CO_2 perception triggers avoidance behavior while in mosquitos it elicits attraction toward the host (Robertson and Kent, 2009; McMeniman et al., 2014). Interestingly, the "domestic" form of *A. aegypti* has evolved host specificity toward humans, and the olfactory coreceptor ORCO is crucial to discriminate human from non-human hosts (DeGennaro et al., 2013). Moreover, this human preference correlates with antennal expression of OR4a, a receptor for the human odorant sulcatone (McBride et al., 2014).

As most animals, flies ingest sugar for nutrition purposes; therefore the ability to taste sweet substances ensures the ingestion of these vital compounds. In the case of the adult *D. melanogaster*, contrary to the larvae, the arrangement of chemoreceptors involved in sugar detection is complex. Several GRs are coexpressed in the same sugar-responding neuron in the labellum and the leg (Fujii et al., 2015). In particular, GR5a expressed in taste neurons detects trehalose, the principal sugar found in the insect's hemolymph (Chyb et al., 2003), and triggers attraction in adults (Thorne et al., 2004; Wang et al., 2004). GR5a, GR61a, and GR64a-f mediate responses to sucrose, maltose, and several other sugars (Dahanukar et al., 2007; Jiao et al., 2007, 2008; Slone et al., 2007; Fujii et al., 2015). GR43a is a fructose receptor in adults but is also necessary for the detection of multiple sugars in larvae (Miyamoto et al., 2012; Mishra et al., 2013). We will discuss in depth the possible rationales behind the complexity of sugar detection in adult flies as well as the differences with the simpler larval system in the Section Chemoreceptors along the Life Cycle: Adult vs. Larvae Dimorphism in Receptors, Structures, and Elicited Behaviors.

In addition to the five canonical taste modalities (sweet, bitter, salt, sour, and umami or the taste of amino acids), flies can detect a range of fatty acids through taste and concomitantly elicit feeding behavior; this represents a clear advantage since fatty acids are a potent energy source for animals. The chemoreceptor dedicated to fatty acids detection in flies remains unknown but fatty acids tasting requires intact phospholipase C signal specifically in sweet-sensing neurons (Masek and Keene, 2013).

Chemoreceptors Involved in Toxic/Bitter Compounds Avoidance

Plants produce a diverse variety of unpalatable compounds as defense mechanisms toward herbivores. These compounds are generally sensed as bitter in the animal taste system and produce an aversive behavior that represents a clear advantage for the plant. Flies, on their behalf, also benefit from this avoidance behavior since many bitter compounds are not very nutritive and are even toxic. Bellow, we will present several examples of toxic/bitter compounds produced by plants (natural insect repellents) that trigger avoidance in flies. In addition, we will also consider the case of DEET, since it is the most widely used synthetic insect repellent nowadays.

One of the first described and most studied receptor for plant bitter compounds in *Drosophila* is that for caffeine. Detection and avoidance of caffeine requires a multimeric receptor including at least GR66a, GR33a, and GR93a subunits (Moon et al., 2006, 2009; Lee et al., 2009). Another plant bitter compound is isothiocyanate, the spicy ingredient of wasabi. Isothiocyanate triggers aversive responses in flies and this repellent behavior depend on the TRP channel Painless (Al-Anzi et al., 2006). Interestingly, this same channel is required for the fructose avoidance behavior that occurs in the change of food attraction to aversion during the wandering stage of larvae (Xu et al., 2008). *Drosophila* flies detect and avoid citronellal, an insect repellent produced by plants, through undescribed olfactory receptors in the antenna. TRPA1 channel is required for this avoidance behavior, and in *A. gambiae* mosquitoes the TRPA1 ortholog responds directly to citronellal (Kwon et al., 2010). Furthermore, many plants can accumulate in their seeds L-canavanine, a toxic amino acid. In flies, L-canavanine triggers strong aversion through the detection by bitter neurons (Mitri et al., 2009). The insect orphan G-protein-coupled DmXR was first identified as the L-canavanine receptor in flies (Mitri et al., 2009), although a later study determined instead GR8a and GR66a to be the chemoreceptors responsible for L-canavanine detection (Lee et al., 2012).

Natural insect repellents are also produced by harmful microorganisms such as *Penicillium* fungal molds and *Streptomyces* soil bacteria. Thanks to the specific olfactory receptor OR56a, flies can detect in the food very small quantities of geosmin, an indicator of contamination with these toxic microorganisms, and avoid the contact with toxic substrates. Through the activation of a dedicated olfactory pathway, geosmin triggers a strong aversive response that includes, oviposition and feeding avoidance, negative taxis, and decreases the attraction toward food odors (Stensmyr et al., 2012).

Although unpleasant, not all of these compounds produced by plants are actually toxic for the flies. An interesting example is the case of camphor, an unpalatable but nontoxic tastant that triggers aversion in adult flies. Very recently, it was demonstrated that pre-exposure to a camphor-rich diet attenuates camphor rejection through reduction of the TRPL receptor expression in the proboscis. In this sense, such desensitization mechanism reduces an unnecessary avoidance of a bitter but non-toxic compound, and this allows the use of camphor rich medium as a nutritional source in the absence of more appealing food sources. Interestingly, when returned to a camphor-free medium flies restore the strong rejection to camphor, suggesting that taste biases could be regulated depending on the quality of available food (Zhang et al., 2013b).

In addition to repulsive chemicals produced by plants, synthetic compounds can also trigger avoidance in flies. In the last 50 years, DEET has been the most widely used synthetic insect repellent. Although it proved to be effective in the control of several insect pests, its mechanisms of action are still under debate. In flies, DEET is detected by GR32a, GR33a, GR66a, and possibly other receptors expressed in GRNs, which mediate the antifeedant effects of the insect repellent (Lee et al., 2010). In addition, DEET inhibits odor-evoked activation of a subset of insect ORs, thereby inhibiting the perception of food odors (Ditzen et al., 2008). In the mosquito *A. aegypti*, DEET acts as an insect repellent at long distances through the activation of ORs. The olfactory detection of DEET not only triggers an immediate aversive response but can also form a short-term aversive memory. Thus, relevant odorants can induce plastic changes in the system, allowing flies to learn to avoid specific substrates (Das et al., 2014).

Finally, sour taste, evoked by low pH and carboxylic acids, is also generally associated with harmful conditions and triggers avoidance. Adult flies generally prefer slightly acid mediums while they reject extremely acid foods (Fuyama, 1976; Ai et al., 2010). While the detection of specific carboxylic acids seems complex and still under debate (see Section Chemoreceptors along the Life Cycle: Adult vs. Larvae Dimorphism in Receptors, Structures, and Elicited Behaviors), adult flies have a simple system to detect protons. IR64 acting together with IR8a form an olfactory receptor to sense acidity in the antenna. The olfactory detection of low pH solutions by the complex IR64a-IR8a activates a dedicated neuronal circuit that leads to avoidance behavior (Ai et al., 2010, 2013).

Chemoreceptors Involved in Oviposition Site Selection

In order to select the proper oviposition site, female flies evaluate the composition of the medium through gustatory receptors present in their ovipositor and proboscis (Yang et al., 2008) as well as olfactory receptors in the antenna (Stensmyr et al., 2012; Dweck et al., 2013). It is believed that in this search, females have to evaluate, according to the presence and concentration of specific chemicals, if larvae will be able to survive or not in this medium. Small quantities of geosmin, an indicator of the presence of harmful microorganisms in a substrate, are detected by OR56a and are sufficient to repel flies from lying eggs on this medium (Stensmyr et al., 2012). In the absence of harmful microorganisms, other chemicals can also prevent fly egg-laying. For instance, *Drosophila* females avoid egg laying in substrates with high sugar concentration, although this decision seems to be highly context dependent (Yang et al., 2008; Schwartz et al., 2012).

The presence of particular chemicals in the substrate can induce oviposition in flies. In this regard, the case of acetic acid is an interesting example. Although both females and males avoid 5% acetic acid solutions (i.e., the concentration present in vinegar), females choose acetic acid supplemented mediums to lay their eggs. The positional avoidance appears to be mediated by olfactory receptors present in the antenna, while the attraction to oviposit depends on gustatory perception (Joseph et al., 2009). In addition, it has been recently demonstrated that terpenes produced by citrus peels, in particular limonene, stimulate oviposition in *Drosophila* through the activation of the OR19a receptor. Interestingly, wasps which parasite *Drosophila* show a strong aversion to these same terpenes, suggesting that oviposition preference on citrus substrate could confer protection against these endoparasitoids (Dweck et al., 2013).

The presence of dedicated olfactory receptors to detect geosmin and limonene allows flies to avoid to oviposit in harmful substrates while promoting oviposition in citrus substrates that will guarantee the absence of wasps parasites. This confers a clear adaptive advantage for *Drosophila* flies, and it suggests an adaptation of the olfactory system to the different substrates present in their natural environment. In contraposition, it remains still unclear why flies prefer to lay eggs in low sugar or acetic acid complemented medium, although some hypotheses have been formulated (Parsons, 1980; Joseph et al., 2009; Schwartz et al., 2012).

Chemoreceptors Involved in Sexual Behavior

Male courtship is a complex and relatively stereotyped behavior that compromises multimodal sensory signals. Males use visual cues to orientate and chase the female, produce auditory signals (known as the "mate song") by wing vibrations, and emit, and perceive through dedicated olfactory and gustatory receptors, many chemical cues (Ziegler et al., 2013). These chemical cues are principally sexual pheromones (Gomez-Diaz and Benton, 2013) although recently it has been demonstrated that food-derived odors can modulate courtship as well (Grosjean et al., 2011). Members of ppk (Liu et al., 2012; Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014), OR (Kurtovic et al., 2007; Van der Goes van Naters and Carlson, 2007; Wang et al., 2011), IR (Koh et al., 2014), and GR (Bray and Amrein, 2003; Miyamoto and Amrein, 2008; Moon et al., 2009; Koganezawa et al., 2010) receptor families have been identified or proposed as sexual pheromones receptors; plus, IR84 is involved in the food-odor-mediated modulation of courtship (Grosjean et al., 2011). In the last few years important advances have been made on the field of chemoreception in sexual behavior, hence in the next section we will present an updated view of the relevance of specific pheromone and food odor receptors involved in courtship. In addition, in the context of chemoreceptors implicated in sexual behavior, we will introduce several examples of sexual

dimorphism in olfactory and gustatory circuits in *Drosophila*. Although we do not intend to go into detail on the differences between male and female chemosensory structures, we hope these examples will serve to illustrate how flies achieve sexual dimorphic behaviors in response to aphrodisiac/anti-aphrodisiac stimuli. Readers interested on sexual dimorphism in *Drosophila* could revise the bibliography proposed in the next section, of which the review of Yamamoto and Koganezawa (2013) is highly recommendable.

Chemoreceptors and Sexual Behavior: Relevance of Pheromone and Food Odor Receptors

OSNs expressing either OR67d, OR47b, or IR84 are the only three OSNs that express a sex-specific transcript of the gene *fruitless* (FruM) (Stockinger et al., 2005; Grosjean et al., 2011), and this suggests their involvement in sex-specific behaviors such as courtship. These OSNs project respectively to DA1, VA1$_v$, and VL2a glomeruli, which are significantly larger in males. From these glomeruli, Fru$^+$ projection neurons connect with the lateral horn (Kondoh et al., 2003; Stockinger et al., 2005; Grosjean et al., 2011). Regarding taste structures, males harbor more gustatory receptors in their legs compared to females, and sex determination factors like *fruitless* and *doublesex* are responsible for sexually dimorphic axonal pattern in these sensory neurons (Possidente and Murphey, 1989; Mellert et al., 2012; Yamamoto and Koganezawa, 2013; Koh et al., 2014) (**Figure 2A**). Fru$^+$ gustatory neurons are present mainly in the dorsal labellum and foreleg tarsi (Stockinger et al., 2005). With the exception of IR84a that has been confirmed to respond to food odors (Grosjean et al., 2011), the rest of these sexually dimorphic receptors are annotated or predicted to detect sexual pheromones.

A sexual pheromone is defined as a chemical signal produced by the organism involved in the control of sexual behaviors. In *Drosophila*, the principal known sexual pheromones are the volatile cis-vaccenyl acetate (cVA) and the cuticular hydrocarbons 7-tricosene (7-T), 7,11-heptacosadiene (7,11-HD), and 7,11 nonacosadiene (7,11-ND). Briefly, cVA and 7-T are produced by males and they act as anti-aphrodisiac for other males (although cVA has several additional roles; see below) while 7,11-HD and 7,11-ND are female pheromones that promote courtship (Fernández and Kravitz, 2013; Gomez-Diaz and Benton, 2013). Anyhow, more recent and highly sensible methods of detection have demonstrated that all of these four pheromones are present in virgin socially naïve individuals of both sexes but in different quantities (Yew et al., 2009). cVA produced and stored in the ejaculatory bulb of males (Butterworth, 1969; Brieger and Butterworth, 1970) is transferred to females during copulation (Butterworth, 1969; Ejima et al., 2007). Together with cVA, an acetylated hydrocarbon named CH503 is also transferred to females during copulation leading to a prolonged inhibition of male courtship acting through an unknown sensory receptor (Yew et al., 2009) (**Figures 2B–D** and **Table 2**).

In males, cVA acts as an anti-aphrodisiac that reduces courtship toward mated females or other males. cVA also modulates male-male aggression while increasing receptivity in females (Jallon, 1984; Ejima et al., 2007; Kurtovic et al., 2007; Wang and Anderson, 2010; Liu et al., 2011). At long ranges, cVA is described to function as an aggregation factor for males and females (Bartelt et al., 1985). Electrophysiological studies demonstrated that cVA is sensed through OSNs expressing OR67d and to a lesser extent, OR65a (Ha and Smith, 2006; Kurtovic et al., 2007; Van der Goes van Naters and Carlson, 2007). Although some discrepancies have been observed in different studies, the role of cVA on sexual behaviors seems to be mediated by OR67d activation (Ejima et al., 2007; Kurtovic et al., 2007). While acute promotion of aggression depends on OR67d, chronic exposure to cVA reduces aggression through OR65a activation (Wang and Anderson, 2010; Liu et al., 2011). Interestingly, both females and males express OR67d and OR65a, and these receptors respond equally to cVA in both sexes (Kurtovic et al., 2007; Van der Goes van Naters and Carlson, 2007). However, the neuronal circuit underlying OR67d is sexually dimorphic and, consequently, different neuronal cluster are activated in males and females (Datta et al., 2008; Ruta et al., 2010; Kohl et al., 2013). This sexual dimorphism in the neuronal circuit downstream of OR67d could be responsible for the different behaviors elicited by cVA in both sexes. In addition to the activation of OR67d and OR65a by cVA, uncharacterized fly odors activate OR47b and OR88a both in males and females, suggesting the presence of other volatile pheromones (Van der Goes van Naters and Carlson, 2007) (**Figures 2B–D** and **Table 2**).

7-T is a male hydrocarbon that inhibits courtship in other males (Antony and Jallon, 1982; Lacaille et al., 2007) and promotes male-male aggression by acting in the same neuronal pathway as cVA (Wang et al., 2011). Several receptors have been proposed for 7-T, in particular GR32a and GR33a (Miyamoto and Amrein, 2008; Moon et al., 2009). Males lacking GR32a display high courtship toward males and mated females, suggesting that GR32a could sense an anti-aphrodisiac signal produced by males and transferred to females during courtship (Miyamoto and Amrein, 2008) (**Figures 2B–D**). Moreover, GR32a prevents males to court with individuals from other species, contributing to the isolation barrier within the *Drosophila* genus (Fan et al., 2013) (**Figure 2E**). GR32a is present in the labellum and in the leg, but only in the leg GR32$^+$ GRNs are surrounded by cells expressing OBP57, an odorant-binding protein implicated in the carrying of pheromones (Koganezawa et al., 2010). Although no sexual dimorphism is observed in GR32a sensory neurons, they seem to contact Fru$^+$ neurons in the SOG that display sexually dimorphic dendritic arbors (Koganezawa et al., 2010; Fan et al., 2013) (**Figure 2A**). It would be interesting to study if these differences in the dendritic arbor determine different postsynaptic neuronal clusters that could activate different motor programs in males and females in response to GR32a activation. At the same time, GR33a, a key receptor in the detection of several aversive compounds, is also required to inhibit male-male courtship (Moon et al., 2009) and it is essential for the male preference for younger virgin females (Hu et al., 2015). GR33a and GR32a seem to be expressed in the same GRNs in the leg, suggesting that they might be part of the same heterodimeric receptor (Moon et al., 2009). In addition, 7-T appears

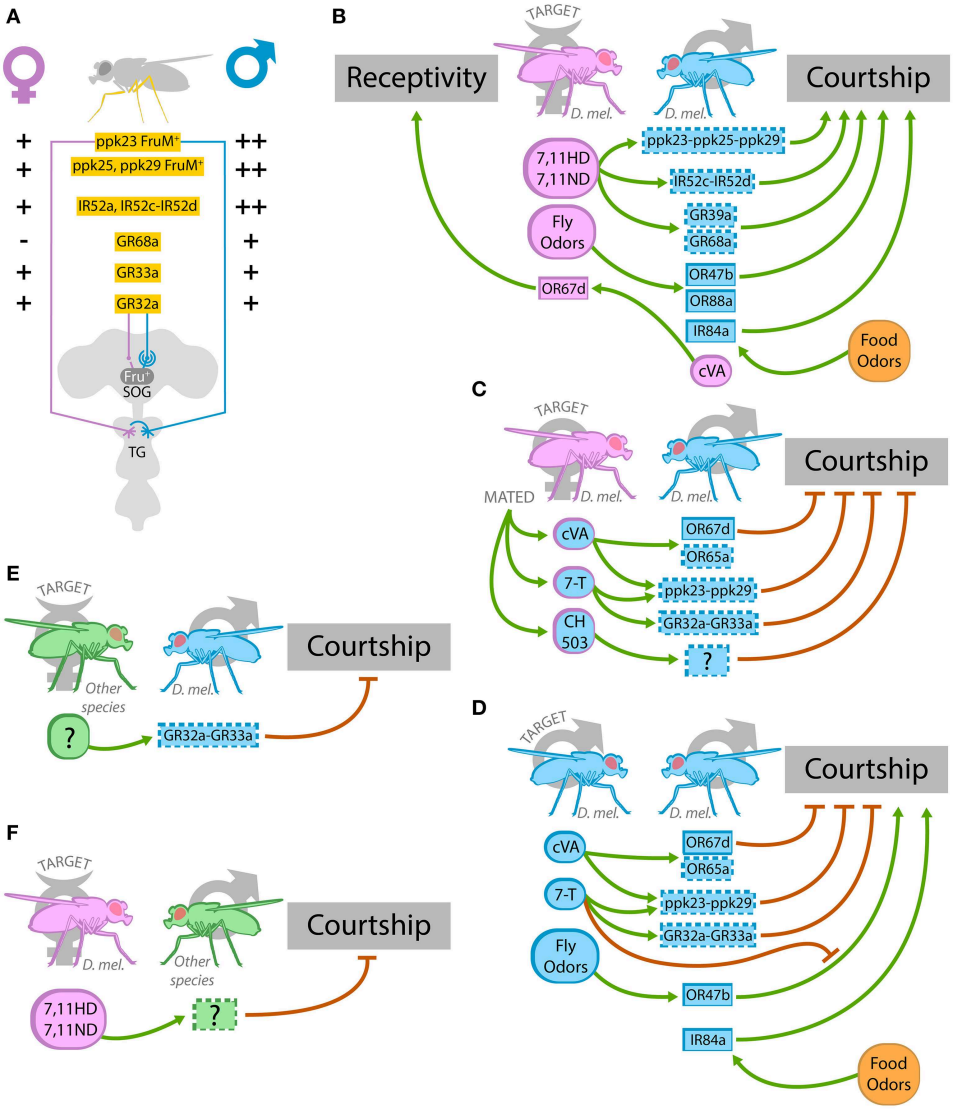

FIGURE 2 | Chemoreceptors involved in sexual behavior. (A) Sexual dimorphism in chemoreceptor's expression and in neuronal morphology of the chemosensory system of the leg. Male legs express more ppk23^{+}, ppk25^{+}, ppk29^{+} FruM^{+}, and IR52a^{+}, IR52c-IR52d^{+} sensory neurons than female legs. In males, axons of ppk23^{+} neurons cross the ventral nerve cord midline in the thoracic ganglion (TG), whereas female axons do not. GR68a is only expressed in male legs, while GR32a and GR33a are expressed in the same number of leg sensory neurons in both sexes. Sexual dimorphism is observed in the dendritic arbor of the GR32a postsynaptic neurons expressing Fru in the SOG. **(B–F)** Illustrations of olfactory and gustatory cues (sexual pheromones or food odors) and the chemoreceptors involved in sexual behavior during an encounter between a virgin female and a male **(B)**, a mated female and a male **(C)**, a male and another male **(D)**, and interspecific heterosexual encounters **(E,F)**. **(B)** Female hydrocarbons 7,11 HD and 7,11 ND activate most likely IR52c-IR52d, GR68a, GR39a, and the complex ppk23-ppk25-ppk29 in the male leg, and this induce courtship. Undetermined fly odors activate OR47b and OR88a, and at least the activation of OR47b leads to increase courtship in males. cVA produced by males stimulates OR67d receptors in female antenna and increase female receptivity. In addition to the signals produced by flies, food odors, in particular phenylacetic acid and phenylacetaldehyde, activate IR84a and promote courtship in males. **(C)** During courtship, males temporarily pass some 7-T hydrocarbon on to females, and cVA and CH503 are transferred from males to females during mating. 7-T deposited on courted females apparently activates GR32a-GR33a and ppk23-ppk29^{+} neurons in the male leg and concomitantly reduces courtship by other males. cVA transferred to mated females activates OR67d and presumably OR65a in the male antenna, and probably ppk23-ppk29^{+} neurons in the male leg, leading to courtship inhibition. CH503 acts through unknown receptors and also leads to a reduction of courtship. **(D)** In a male-male encounter, cVA and 7-T inhibit homosexual courtship acting through OR67d, OR65a in the antenna and presumably ppk23-ppk29, GR32a-GR33a complexes in the leg. The courtship-promoting signal that follows the activation of OR47b by fly odors is inhibited by 7-T. Food odors activate IR84a and promote male-male courtship. To simplify, only the signals emitted by one of the males are shown in the drawing but the reciprocal ones are also present in the encounter. **(E)** Unidentified cuticular hydrocarbons of females of other *Drosophila* species (*D. simulans*, *D. yakuba*, and *D. virilis*) are most likely sensed by GR32a-Gr33a in *D. melanogaster* males and this prevents interspecific courtship. **(F)** The female hydrocarbons 7,11 HD and 7,11 ND act through unknown chemoreceptors in males of other *Drosophila* species (*D. simulans*, *D. yakuba*, and *D. erecta*) and inhibit courtship. In panels **(B–F)**, pink stands for female, blue, male, and green, male or female of another *Drosophila* species. In the boxes, a full-lined frame indicates that the protein is a *bona fide* chemoreceptor, while a dotted-line means that there is still no clear demonstration of the protein's role as chemoreceptor.

to inhibit a male-male courtship-promoting-signaling pathway that is OR47b dependent (Wang et al., 2011) (**Figures 2B–E** and **Table 2**).

The production of 7,11 HD in females serves as an aphrodisiac for males of the same species (Antony and Jallon, 1982; Antony et al., 1985) and acts as a barrier to prevent interspecific courtship (Billeter et al., 2009). Males sense female pheromones, probably 7,11-HD, through GR68a expressed in their forelegs (**Figures 2B,F** and **Table 2**). GR68a is exclusively expressed in male forelegs (**Figure 2A**), and its expression depends on the sex determination factor *doublesex* (Bray and Amrein, 2003). In addition, GR39a may also be involved in female pheromones perception since male mutants for GR39a display reduced courtship toward wild-type females (Watanabe et al., 2011).

In the last few years, several studies have demonstrated the relevance of ppk channels, notably ppk23, ppk25, and ppk29, in sexual behavior. These 3 channels are expressed in Fru^+ gustatory neurons of both sexes although males have around double the amount of ppk^+ cells in the leg compared to females (Liu et al., 2012; Lu et al., 2012; Vijayan et al., 2014) (**Figure 2A**). Different subpopulations of $ppk23^+$-$FruM^+$ neurons in the leg respond to male and female pheromones, and both ppk23 and ppk29 are required for the pheromone-evoked effects on courtship (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012). Those responding to female pheromones express also ppk25, and this channel is necessary for the 7,11-HD effects of promoting courtship toward virgin females as well as for the stimulation of courtship by pheromones present on immature males and for normal female receptivity (Vijayan et al., 2014) (**Figures 2B–D** and **Table 2**). ppk25 is also expressed in olfactory neurons, but this expression is not relevant for courtship control (Starostina et al., 2012). Interestingly, similar responses at the level of ppk^+ cells activation in response to male and female compounds were observed in both sexes (Thistle et al., 2012; Vijayan et al., 2014). This suggests, once again, that sexual dimorphism downstream of the activation of receptor neurons might be responsible for the different behaviors triggered in male and females in response to sexual pheromones. In effect, at least $ppk23^+$ neurons display sexually dimorphic axonal projections, although the physiological consequences of this sexual dimorphism have not been studied (Lu et al., 2012; Thistle et al., 2012) (**Figure 2A**).

The studies presented here demonstrate the relevance of ppk23, ppk25, and ppk29 channels in sexual behavior, but a clear demonstration of their role as chemoreceptors is still lacking. Attempts to prove the direct requirement of ppk23 and ppk29 as pheromone receptors failed, suggesting that additional subunits may be required (Thistle et al., 2012). Consistent with this idea is the fact that ppk29 (also known as NOPE) forms a complex with ppk25 (Liu et al., 2012). Alternatively, ppk channels could be playing a fundamental role on pheromone-evoked responses, not as direct receptors but as a unique cellular component of gustatory neurons expressing a yet unknown chemoreceptor (Pikielny, 2012). Strikingly, a recent study demonstrated that ppk23 and ppk29 are also essential for the detection of a novel aggregation pheromone in *D. melanogaster* larvae, the (*Z*)-5 and (*Z*)-7-tetradecenoic acid (Mast et al., 2014). In this study the authors clearly demonstrate the relevance of ppk23 and ppk29 in the detection of the aggregation pheromone but, again, no direct proof of their role as chemoreceptors has been provided. Taking into account that ppk23-ppk29 are essential for the detection of signals of very different structure (long-chain fatty acids in the case of larval aggregation and hydrocarbons in the case of sexual behavior), it seems more reasonable that they don't act as direct chemoreceptors. Anyhow, more suitable experiments like analysis of response to pheromones using *in vitro* or *in vivo* ectopic expression of these proteins will help to clarify this matter.

In addition to ORs, GRs, and ppk channels, IRs play a relevant role in the control of sexual behaviors in *Drosophila*. IR52a, IR52c, and IR52d are present in the foreleg of both sexes although they are expressed in more cells in males (**Figure 2A**). IR52c and IR52d show complete, or nearly complete, coexpression in the foreleg suggesting that they are part of the same complex. $IR52c^+$ neurons are activated by female compounds and form putative synapses with Fru^+ neurons in the prothoracic ganglia. Interestingly, ectopic activation of $IR52c^+$ neurons increase courtship while mutants lacking IR52c and IR52d display reduced courtship behavior and higher latency to copulate, suggesting a possible role on sexual pheromone detection (Koh et al., 2014) (**Figure 2B** and **Table 2**).

Last but not least, food odors, notably phenylacetaldehyde and phenylacetic acid, can also promote male courtship through IR84a-$FruM^+$ OSNs. The VL2a $FruM^+$ glomerulus is activated downstream of $IR84a^+$ OSNs, and from this glomerulus, projection neurons send olfactory information to a pheromone-processing region of the lateral horn (Grosjean et al., 2011) (**Figures 2B,D** and **Table 2**). In this regard, we now understand that not only sexual pheromones but also compounds present in the environment, at least in the fly food, directly modulate sexual behavior in *Drosophila*, highlighting the impact of external cues in a key behavior for species survival.

Chemoreceptors along the Life Cycle: Adult vs. Larvae Dimorphism in Receptors, Structures, and Elicited Behaviors

When we compare a *Drosophila* larva with an adult, differences become much more obvious than similarities. Although sharing the same genome and developmental program, the larval and adult stages of holometabolous insects contrast strikingly in regards to general anatomy, behaviors displayed, and lifestyles or niches occupied. In the nervous system, the differences between larvae and adults rise as a consequence of the extensive apoptosis and neuronal remodeling occurring during the metamorphosis (Truman, 1990). The case of chemoreceptive structures is not an exception. The external taste organs of the larva, the terminal and ventral organs, undergo apoptosis during the metamorphosis and are then completely replaced by adult structures. While the main olfactory organ, the dorsal organ, does not disappear during metamorphosis, the olfactory system undergoes critical neuronal changes, e.g., neuronal migration, proliferation, and development of progenitor cells, dendritic pruning and extension, and axonal remodeling, among other processes (Gerber and Stocker, 2007; Rodrigues and Hummel, 2008). In this regard,

taking into account the extensive remodeling of the chemoreceptive structures along the life cycle of the fly, it does not come as a surprise that larvae and adults can trigger very different behaviors in response to the same stimuli or that they may even use different receptors to detect the same compounds. Nonetheless, one can still wonder why invest so much energy in developing two strikingly different chemoreception systems in such a short time. Are these differences a consequence of developmental constrains or do they reflect an adaptation to different niches occupied along the life? Below, we will present the cases of sugar, carboxylic acid, and salt detection in adult and larvae as interesting examples of stage-specific chemoreceptors involved in the responses to ecologically relevant compounds. We will further discuss how these differences in the system and the behavior could be interpreted in the context of stage-specific needs.

Sugar detection is an interesting example of the differences between larval and adult taste systems. In adults, responses to common sugars, like glucose, sucrose, and maltose, may include up to eight gustatory receptors, i.e., GR5a, GR61a, and GR64a-f (Dahanukar et al., 2007; Jiao et al., 2007, 2008; Slone et al., 2007; Fujii et al., 2015). Fructose is detected by GR43a, a narrowly tuned receptor expressed in taste organs as well as in the central nervous system (**Table 2** and **Figure 3A**). After a sugar-rich diet, the levels of glucose and trehalose in hemolymph do not increase significantly while fructose levels increase between 3 and 10 times. In line with this, GR43a located in the brain acts as a nutrient sensor, assessing the levels of fructose in the hemolymph (Miyamoto et al., 2012). Even though larvae detect and behaviorally respond to sugar, none of the sweet receptors GR5a, GR61a, and GR64a-f are expressed in this stage (Kwon et al., 2011). Alternatively, larvae only express GR43a in internal taste neurons and in the brain. Larvae display an immediate attraction to fructose and sucrose (disaccharide of fructose and glucose) and a delayed preference toward glucose and trehalose (disaccharide of glucose). Surprisingly, all of these attractive responses depend on GR43a; fructose's attraction depends on GR43a expressed in internal taste neurons while glucose's attraction requires GR43a in the brain (Mishra et al., 2013). These differences in dynamics and receptor localization, suggest that larvae sense fructose or fructose-containing disaccharides directly on internal pharyngeal taste neurons while detection of non-fructose sugars relies on their conversion to fructose post-ingestion, elevation of fructose

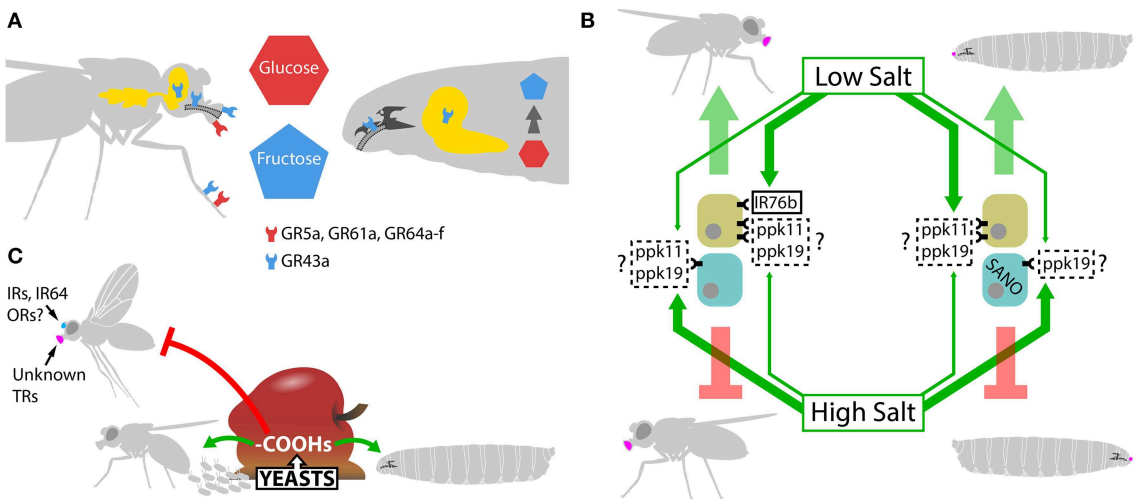

FIGURE 3 | Adult vs. larvae dimorphism. (A) Sugar detection mechanisms in adult and *Drosophila* larvae. In adults, glucose, sucrose, and other common sugars are sensed in the periphery by GR5a, GR61a, and GR64a-f, while fructose activates specifically GR43a in the labellum, legs, internal taste organs, and in the brain. GR43a located in the brain acts as a nutrient sensor, assessing the levels of fructose in the hemolymph. In larvae, only GR43a is expressed. This sugar receptor is present in internal taste organs where it is responsible for fructose or fructose-containing disaccharides detection in the feeding medium, and in the brain where it assesses internal fructose levels. Non-fructose sugars are not detected in the periphery, their detection depends on their internal conversion to fructose and subsequent sensing of internal fructose levels by GR43a in the brain. **(B)**. Carboxylic acid (-COOHs) perception. Rotten fruits present moderate concentrations of different carboxylic acids product of yeast and bacteria fermentation. The presence of these carboxylic acids induces oviposition and, at the same time, triggers positional avoidance in adults. On the contrary, larvae are attracted by carboxylic acid rich media. The chemoreceptors implied in these responses are still not well defined, but at least in adults, IRs, ORs, and the H^+ sensor IR64a in the olfactory system and unknown taste receptors (TR) are responsible for carboxylic acid perception. **(C)** Salt detection in adult and *Drosophila* larva. Both adults and larvae are attracted to low salt and repulsed by high-salt solutions. In adults, IR76b is a Na^+-permeable channel essential for low-salt responses; plus, ppk11 and ppk19 might be involved in the attraction toward low-salt solutions. IR76b is expressed in a subset of GRNs, which respond strongly to low salt, while a different subpopulation of GRNs is strongly activated by high-salt solutions. In larvae, ppk11 and ppk19 are most likely the taste receptors responsible for attractive responses to low-salt concentrations. The chemoreceptors for high-salt solutions remain unknown, but ppk11 and ppk19 may contribute to the responses. At least in larvae, the cytoplasmic protein SANO expressed in bitter neurons is essential for the behavioral aversion to high-salt concentrations. The thick green arrows indicate strong activation while the thin ones, weak activation. In the boxes, a full-lined frame indicates that the protein is a *bona fide* chemoreceptor, while a dotted-line means that there is still no clear demonstration of the protein's role as chemoreceptor.

levels in the hemolymph, and subsequent sensing in the brain (Mishra et al., 2013) (**Table 2** and **Figure 3A**). This apparently inefficient sugar sensing setup provides a simple system that satisfies the larval needs. In order to grow, larvae need to constantly incorporate nutrients and, since their mobility is reduced, looking for the perfect sugar-source could result in great energy costs. It is the adult female fly who carefully analyzes the composition of the medium before choosing an oviposition site and, in doing so, it seems to look for a suitable substrate that will provide with the minimal nutritional requirements for the larvae to grow (Joseph et al., 2009; Schwartz et al., 2012). In addition, fructose and sucrose are present in most fruits, suggesting that having only a rapid fructose detection system may be sufficient on most ecologically relevant substrates. In this sense, larvae generally don't need to search for sugars but they can simply start eating and then evaluate the nutritional content by fast activation of pharyngeal receptors or slower activation of brain receptors. Adult flies, on the contrary, display more complex behaviors that involve the exploration of more heterogeneous environments. In these new environments, flies not only need to evaluate the substrates for the presence of sugars but also the quality of those sugars, since non-fructose sugars may be present in higher proportion. In this sense, a more complex taste system allowing rapid detection of a huge variety of sugars appears as a more suitable setup than the simple version of the larvae.

Another interesting example of chemoreception dimorphism in the fly's life cycle is the case of carboxylic acids perception. While adult flies are strongly repulsed by acidity or high carboxylic acid concentrations (Fuyama, 1976; Ai et al., 2010), larvae display clear attraction (Monte et al., 1989; Kreher et al., 2008; Khurana and Siddiqi, 2013). The chemoreceptors relevant for the attractive responses in larvae have not been identified yet, and only weak activation of some ORs in response to carboxylic acids has been observed (Kreher et al., 2005, 2008). In the case of adults, carboxylic acids are detected through olfaction and taste. In the olfactory system, protons are directly detected by the complex IR64-IR8a (Ai et al., 2010, 2013), and different carboxylic acids trigger strong electrophysiological responses in IR8a$^+$ neurons and mild responses in OR neurons (Hallem and Carlson, 2006; Abuin et al., 2011; Silbering et al., 2011). Nevertheless, efforts to elucidate the role of IR8a on carboxylic acid-triggered behavioral avoidance produced contradictory results (Silbering et al., 2011; Ai et al., 2013). In the gustatory system, it was recently shown that high concentrations of carboxylic acids activate a subset of bitter neurons while they inhibit the activity of sweet neurons (Charlu et al., 2013). At the same time, another study demonstrated that carboxylic acids suppress bitter neuron activity when presented in dietary relevant concentrations (Chen and Amrein, 2014). Interestingly, normally aversive bitter/sugar mixtures are rendered more appealing with the addition of moderate concentrations of carboxylic acids (Chen and Amrein, 2014). The identity of the carboxylic acid receptors in the taste organ has not been revealed yet, but they seem to be different from the H^+ sensor IR64a and the bitter receptors GR33a and Painless (Charlu et al., 2013) (**Table 2** and **Figure 3B**).

Several carboxylic acids are normally present in fly food as fermentation products of yeast and bacteria (Bridges and Mattice, 1939; Idstein et al., 1985; Moat et al., 2002) so, in addition to a simple pH indicator, detection of high concentration of carboxylic acids may serve also as indication of rotten fruit and of the presence of yeasts. This would be important because a previously processed substrate such as rotten fruit could be easier for larvae to feed on; plus, yeasts are the typical source of important nutrients for the larvae, such as proteins and some carbohydrates (Lee et al., 2008; Schwarz et al., 2014). On the other hand, extremely acid solutions are very toxic for adult flies and can result in high mortality in the population (Chakir et al., 1993). The resistance to high concentration of carboxylic acids observed in larvae could represent a tolerance product of the long exposure to low pH media. Consistent with this idea is the fact that female flies normally lay eggs in rotting fruit (Atkinson and Shorrocks, 1977; Markow, 1988), suggesting that larvae are exposed to low pH media throughout their development (**Figure 3B**). In adults, tolerance to high acetic acid concentrations has already been described for a geographic population. Although still unclear, this increased tolerance could be a consequence of a more efficient detoxification system (Chakir et al., 1993). It would be interesting to test if larvae also have a more efficient detoxification system that allows them to tolerate long exposures to high concentrations of carboxylic acids present in their environment.

Animals in general present bimodal responses to salts: low concentrations of salt trigger attraction while high concentrations, repulsion. This feature reflects the dual effect of salt in the organism: moderate levels of salt are necessary to control electrolyte homeostasis, neuronal activity, and muscle contraction while high levels have deleterious effects as dehydration and hypertension (Liman et al., 2014). In *Drosophila*, larvae and adult also display the same bimodal responses to salts (Miyakawa, 1981; Balakrishnan and Rodrigues, 1991) but they detect salt apparently through different mechanisms. Attractive responses to low-salt concentrations in larvae require the ENac channels ppk11 and ppk19 (Liu et al., 2003b) in taste neurons. Interestingly, ENac channels are also involved in the low-salt responses in mammals (Chandrashekar et al., 2010), albeit there is no consistent proof that ppk11 and ppk19 in fly larvae or ENac channels in mammals are direct receptors for low-salt solutions (Liu et al., 2003b; Chandrashekar et al., 2010). In adult flies, a recent paper described IR76b as a Na^+-permeable channel essential for low-salt responses. Furthermore, the authors have clearly demonstrated the existence of two populations of GRNs, one displaying a stronger response to high-salt concentrations and the other one, expressing IR76b, displaying a stronger response to low-salt concentrations. IR76b$^+$ taste neurons constitute a new class of GRN specifically tuned to low-salt detection (Zhang et al., 2013a). Interestingly, IR76b is also present in some adult antennal coeloconic OSNs where it might act as a coreceptor (Silbering et al., 2011). In addition to IR76b, ppk11 and ppk19 might also play a role in low-salt detection in adults (Liu et al., 2003b). Coimmunostaining analysis would help to elucidate if ppk11, ppk19, and IR76b are all part of the same detection system or if they constitute two parallel pathways. Moreover, future experiments should analyze whether IR76b is also required in larvae for low-salt detection (**Table 2** and **Figure 3C**).

Regarding high-salt detection, the receptors are still elusive, but ppk11 and ppk19 may contribute to the aversive responses in both adults and larvae (Liu et al., 2003b; Alves et al., 2014). A recent study identified Serrano (SANO), a cytoplasmic protein expressed in bitter neurons, as an essential molecule for the behavioral aversion to high-salt concentrations in larvae. Moreover, inactivation of $SANO^{+}$ bitter neurons triggers an attractive response to high-salt concentrations in larvae (Alves et al., 2014). This strongly suggests that, as it is the case in adults, two neuronal groups are simultaneously activated in response to both low and high-salt concentration, but it is the outcome between these two populations what will determine if there is attraction or repulsion (**Figure 3C**). Again, the requirement of SANO for high-salt detection in adults was not analyzed. Complementary studies are needed to clearly define whether larvae and adult salt-detection systems are conserved or not.

Perspectives

Our current knowledge of chemosensory perception in *D. melanogaster* is growing very fast with the identification and ongoing characterization of the different receptor families. However, there is still a lot to do to clearly understand how chemicals are detected, and how this information is processed at the periphery and in the brain to lead to a specific behavioral response. For example, most studies focus on identifying potential ligands for a specific chemoreceptor by using single odor stimulation, a case far removed from the complexity of the natural environmental conditions to which flies are normally exposed. In the natural environment odors are generally present in complex mixtures and it is from these blends that flies need to extract the most relevant signals to behave accordingly. Some putative mechanisms for how the olfactory system decodes relatively complex odorant mixtures have been proposed (Silbering and Galizia, 2007) but this still remains a very important open question.

The complexity of sensing and decoding chemical mixtures is also true for taste perception. It has been shown in a recent study (Chen and Amrein, 2014) that the presence of carboxylic acids in a mixture can modulate bitter and sweet perception. Several questions arise from this observation, could the activation of a specific neuron sensitive to acids potentiate the activity of the neighboring sugar sensing neurons in the peripheral nervous system? Is this possible mechanism shared by all chemosensory neurons? Or could it be specific to some neurons and sensory modalities? Moreover, could acids also inhibit bitter sensing neurons as suggested by this recent work (Chen and Amrein, 2014)?

Concerning the integration of the chemosensory stimuli in the brain the picture is still incomplete. Even though the olfactory system is better described than the gustatory system their neuronal networks are still under characterization. What are the exact connections between the different centers in the brain? Moreover, the precise and complete network from the detection of a chemical at the periphery to the muscle cells that lead to a behavioral output is still partially described. Some recent studies on cVA detection have started to decipher this network (Kohl et al., 2013), and have shown that this cVA circuit seems to be interconnected with other sensory modalities such as hearing (Zhou et al., 2014) which highlights the importance of the connectivity between modalities.

D. melanogaster is a powerful genetic model and we owe it most of our current knowledge on the molecular basis of chemoreception in insects. Nonetheless, it would be interesting to compare how chemical perception is processed in other *Drosophila* species that have a highly specialized living substrate and to analyze differences and similarities between them. Through these comparative studies we could follow evolutionary traces and study if specific sensory systems have been selected to ensure species survival. The comparison with the chemosensory systems of more distant insects such as mosquitos and bees would also be of great value for the management of species that impact deeply on human health and agriculture.

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References

Abuin, L., Bargeton, B., Ulbrich, M. H., Isacoff, E. Y., Kellenberger, S., and Benton, R. (2011). Functional architecture of olfactory ionotropic glutamate receptors. *Neuron* 69, 44–60. doi: 10.1016/j.neuron.2010.11.042

Ai, M., Blais, S., Park, J. Y., Min, S., Neubert, T. A., and Suh, G. S. (2013). Ionotropic glutamate receptors IR64a and IR8a form a functional odorant receptor complex *in vivo* in Drosophila. *J. Neurosci.* 33, 10741–10749. doi: 10.1523/JNEUROSCI.5419-12.2013

Ai, M., Min, S., Grosjean, Y., Leblanc, C., Bell, R., Benton, R., et al. (2010). Acid sensing by the Drosophila olfactory system. *Nature* 468, 691–695. doi: 10.1038/nature09537

Al-Anzi, B., Tracey, W. D., and Benzer, S. (2006). Response of Drosophila to wasabi is mediated by painless, the fly homolog of mammalian TRPA1/ANKTM1. *Curr. Biol.* 16, 1034–1040. doi: 10.1016/j.cub.2006.04.002

Alves, G., Sallé, J., Chaudy, S., Dupas, S., and Manière, G. (2014). High-NaCl perception in *Drosophila melanogaster*. *J. Neurosci.* 34, 10884–10891. doi: 10.1523/JNEUROSCI.4795-13.2014

Antony, C., and Jallon, J. (1982). The chemical basis for sex recognition in *Drosophila melanogaster*. *J. Insect Physiol.* 28, 873–880. doi: 10.1016/0022-1910(82)90101-9

Antony, C., Davis, T., Carlson, D., Pechine, J., and Jallon, J. (1985). Compared behavioral responses of male *Drosophila melanogaster* (Canton S) to natural and synthetic aphrodisiacs. *J. Chem. Ecol.* 11, 1617–1629. doi: 10.1007/BF01012116

Asahina, K., Pavlenkovich, V., and Vosshall, L. B. (2008). The survival advantage of olfaction in a competitive environment. *Curr. Biol.* 18, 1153–1155. doi: 10.1016/j.cub.2008.06.075

Atkinson, W., and Shorrocks, B. (1977). Oecologia in the domestic species of Drosophila. *Oecologia* 29, 223–232. doi: 10.1007/BF00345697

Balakrishnan, B. Y. R., and Rodrigues, V. (1991). The shaker and shaking-B genes specify elements in the processing of gustatory information in *Drosophila melanogaster*. *J. Exp. Biol.* 157, 161–181.

Bartelt, R. J., Schaner, A. M., and Jackson, L. L. (1985). cis-Vaccenyl acetate as an aggregation pheromone in *Drosophila melanogaster*. *J. Chem. Ecol.* 11, 1747–1756. doi: 10.1007/BF01012124

Becher, P. G., Bengtsson, M., Hansson, B. S., and Witzgall, P. (2010). Flying the fly: long-range flight behavior of *Drosophila melanogaster* to attractive odors. *J. Chem. Ecol.* 36, 599–607. doi: 10.1007/s10886-010-9794-2

Benton, R., Sachse, S., Michnick, S. W., and Vosshall, L. B. (2006). Atypical membrane topology and heteromeric function of Drosophila odorant receptors *in vivo*. *PLoS Biol.* 4:e20. doi: 10.1371/journal.pbio.0040020

Benton, R., Vannice, K. S., Gomez-diaz, C., and Vosshall, L. B. (2009). Variant ionotropic glutamate receptors as chemosensory receptors in Drosophila. *Cell* 136, 5–7. doi: 10.1016/j.cell.2008.12.001

Billeter, J.-C., Atallah, J., Krupp, J. J., Millar, J. G., and Levine, J. D. (2009). Specialized cells tag sexual and species identity in *Drosophila melanogaster*. *Nature* 461, 987–991. doi: 10.1038/nature08495

Boll, W., and Noll, M. (2002). The Drosophila Pox neuro gene: control of male courtship behavior and fertility as revealed by a complete dissection of all enhancers. *Development* 129, 5667–5681. doi: 10.1242/dev.00157

Bray, S., and Amrein, H. (2003). A putative Drosophila pheromone receptor expressed in male-specific taste neurons is required for efficient courtship. *Neuron* 39, 1019–1029. doi: 10.1016/S0896-6273(03)00542-7

Bridges, M. A., and Mattice, M. R. (1939). Over two thousand estimations of the ph of representative foods. *Am. J. Dig. Dis.* 6, 440–449. doi: 10.1007/BF02996505

Brieger, G., and Butterworth, F. M. (1970). *Drosophila melanogaster*: identity of male lipid in reproductive system. *Science* 167:1262. doi: 10.1126/science.167.3922.1262

Butterworth, F. M. (1969). Lipids of Drosophila: a newly detected lipid in the male. *Science* 163, 1356–1357. doi: 10.1126/science.163.3873.1356

Cameron, P., Hiroi, M., Ngai, J., and Scott, K. (2010). The molecular basis for water taste in Drosophila. *Nature* 465, 91–95. doi: 10.1038/nature09011

Cande, J., Prud'homme, B., and Gompel, N. (2013). Smells like evolution: the role of chemoreceptor evolution in behavioral change. *Curr. Opin. Neurobiol.* 23, 152–158. doi: 10.1016/j.conb.2012.07.008

Chakir, M., Peridy, O., Capy, P., Pla, E., and David, J. R. (1993). Adaptation to alcoholic fermentation in Drosophila: a parallel selection imposed by environmental ethanol and acetic acid. *Proc. Natl. Acad. Sci. U.S.A.* 90, 3621–3625. doi: 10.1073/pnas.90.8.3621

Chandrashekar, J., Hoon, M. A., Ryba, N. J. P., and Zuker, C. S. (2006). The receptors and cells for mammalian taste. *Nature* 444, 288–294. doi: 10.1038/nature05401

Chandrashekar, J., Kuhn, C., Oka, Y., Yarmolinsky, D. A., Hummler, E., Ryba, N. J., et al. (2010). The cells and peripheral representation of sodium taste in mice. *Nature* 464, 297–301. doi: 10.1038/nature08783

Charlu, S., Wisotsky, Z., Medina, A., and Dahanukar, A. (2013). Acid sensing by sweet and bitter taste neurons in *Drosophila melanogaster*. *Nat. Commun.* 4:2042. doi: 10.1038/ncomms3042

Chen, Y., and Amrein, H. (2014). Enhancing perception of contaminated food through acid-mediated modulation of taste neuron responses. *Curr. Biol.* 24, 1969–1977. doi: 10.1016/j.cub.2014.07.069

Chyb, S., Dahanukar, A., Wickens, A., and Carlson, J. R. (2003). Drosophila Gr5a encodes a taste receptor tuned to trehalose. *Proc. Natl. Acad. Sci. U.S.A.* 100(Suppl.), 14526–14530. doi: 10.1073/pnas.2135339100

Clyne, P. J. (2000). Candidate Taste Receptors in Drosophila. *Science.* 287, 1830–1834. doi: 10.1126/science.287.5459.1830

Clyne, P. J., Warr, C. G., Freeman, M. R., Lessing, D., Kim, J., and Carlson, J. R. (1999). A Novel family of Divergent Seven-Transmembrane proteins?: candidate odorant receptors in Drosophila. *Neuron* 22, 327–338. doi: 10.1016/S0896-6273(00)81093-4

Colomb, J., Grillenzoni, N., Ramaekers, A., and Stocker, R. F. (2007). Architecture of the primary taste center of *Drosophila melanogaster* larvae. *J. Comp. Neurol.* 847, 834–847. doi: 10.1002/cne.21312

Couto, A., Alenius, M., and Dickson, B. J. (2005). Molecular, anatomical, and functional organization of the Drosophila olfactory system. *Curr. Biol.* 15, 1535–1547. doi: 10.1016/j.cub.2005.07.034

Croset, V., Rytz, R., Cummins, S. F., Budd, A., Brawand, D., Kaessmann, H., et al. (2010). Ancient protostome origin of chemosensory ionotropic glutamate receptors and the evolution of insect taste and olfaction. *PLoS Genet.* 6:e1001064. doi: 10.1371/journal.pgen.1001064

Dahanukar, A., Foster, K., van der Goes van Naters, W. M., and Carlson, J. R. (2001). A Gr receptor is required for response to the sugar trehalose in taste neurons of Drosophila. *Nat. Neurosci.* 4, 1182–1186. doi: 10.1038/nn765

Dahanukar, A., Lei, Y.-T., Kwon, J. Y., and Carlson, J. R. (2007). Two Gr genes underlie sugar reception in Drosophila Anupama. *Neuron* 56, 503–516. doi: 10.1016/j.neuron.2007.10.024

Dambly-chaudière, C., and Ghysen, A. (1986). The sense organs in the Drosophila larva and their relation to the embryonic pattern of sensory neurons. *Roux's Arch. Dev. Biol.* 195, 222–228. doi: 10.1007/BF02438954

Das, G., Klappenbach, M., Vrontou, E., Perisse, E., Clark, C. M., Burke, C. J., et al. (2014). Drosophila learn opposing components of a compound food stimulus. *Curr. Biol.* 24, 1723–1730. doi: 10.1016/j.cub.2014.05.078

Datta, S. R., Vasconcelos, M. L., Ruta, V., Luo, S., Wong, A., Demir, E., et al. (2008). The Drosophila pheromone cVA activates a sexually dimorphic neural circuit. *Nature* 452, 473–477. doi: 10.1038/nature06808

DeGennaro, M., McBride, C. S., Seeholzer, L., Nakagawa, T., Dennis, E. J., Goldman, C., et al. (2013). Orco mutant mosquitoes lose strong preference for humans and are not repelled by volatile DEET. *Nature* 498, 487–491. doi: 10.1038/nature12206

Ditzen, M., Pellegrino, M., and Vosshall, L. B. (2008). Insect odorant receptors are molecular targets of the insect repellent DEET. *Science* 319, 1838–1842. doi: 10.1126/science.1153121

Dobritsa, A. A., Van der Goes van Nater, W., Warr, C. G., Steinbrecht, R. A., and Carlson, J. R. (2003). Integrating the molecular and cellular basis of Odor coding in the Drosophila Antenna. *Neuron* 37, 827–841. doi: 10.1016/S0896-6273(03)00094-1

Dunipace, L., Meister, S., McNealy, C., and Amrein, H. (2001). Spatially restricted expression of candidate taste receptors in the Drosophila gustatory system. *Curr. Biol.* 11, 822–835. doi: 10.1016/S0960-9822(01)00258-5

Dweck, H. K. M., Ebrahim, S. A. M., Farhan, A., Hansson, B. S., and Stensmyr, M. C. (2015). Olfactory proxy detection of dietary antioxidants in Drosophila. *Curr. Biol.* 25, 455–466. doi: 10.1016/j.cub.2014.11.062

Dweck, H. K. M., Ebrahim, S. A. M., Kromann, S., Bown, D., Hillbur, Y., Sachse, S., et al. (2013). Olfactory preference for egg laying on citrus substrates in Drosophila. *Curr. Biol.* 23, 2472–2480. doi: 10.1016/j.cub.2013.10.047

Ejima, A., Smith, B. P. C., Lucas, C., van der Goes van Naters, W., Miller, C. J., Carlson, J. R., et al. (2007). Generalization of courtship learning in Drosophila is mediated by cis-vaccenyl acetate. *Curr. Biol.* 17, 599–605. doi: 10.1016/j.cub.2007.01.053

Fan, P., Manoli, D. S., Ahmed, O. M., Chen, Y., Agarwal, N., Kwong, S. et al. (2013). Genetic and neural mechanisms that inhibit Drosophila from mating with other species. *Cell* 154, 89–102. doi: 10.1016/j.cell.2013.06.008

Faucher, C., Forstreuter, M., Hilker, M., and de Bruyne, M. (2006). Behavioral responses of Drosophila to biogenic levels of carbon dioxide depend on life-stage, sex and olfactory context. *J. Exp. Biol.* 209, 2739–2748. doi: 10.1242/jeb.02297

Fernández, M. P., and Kravitz, E. A. (2013). Aggression and courtship in Drosophila: pheromonal communication and sex recognition. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 199, 1065–1076. doi: 10.1007/s00359-013-0851-5

Fischler, W., Kong, P., Marella, S., and Scott, K. (2007). The detection of carbonation by the Drosophila gustatory system. *Nature* 448, 1054–1057. doi: 10.1038/nature06101

Fishilevich, E., and Vosshall, L. B. (2005). Genetic and functional subdivision of the Drosophila antennal lobe. *Curr. Biol.* 15, 1548–1553. doi: 10.1016/j.cub.2005.07.066

Fishilevich, E., Domingos, A. I., Asahina, K., Naef, F., Vosshall, L. B., Louis, M., et al. (2005). Chemotaxis behavior mediated by single larval olfactory neurons in Drosophila. *Curr. Biol.* 15, 2086–2096. doi: 10.1016/j.cub.2005.11.016

Fowler, M. A., and Montell, C. (2013). Drosophila TRP channels and animal behavior. *Life Sci.* 92, 394–403. doi: 10.1016/j.lfs.2012.07.029

Fujii, S., Yavuz, A., Slone, J., Jagge, C., Song, X., Amrein, H., et al. (2015). Drosophila sugar receptors in sweet taste perception, olfaction, and internal nutrient sensing. *Curr. Biol.* 25, 621–627. doi: 10.1016/j.cub.2014.12.058

Fuyama, Y. (1976). Behavior genetics of olfactory responses in Drosophila. *I.* Oifactometry and strain differences in *Drosophila melanogaster*. *Behav. Genet.* 6, 406–420. doi: 10.1007/BF01065698

Gerber, B., and Stocker, R. F. (2007). The Drosophila larva as a model for studying chemosensation and chemosensory learning: a review. *Chem. Senses* 32, 65–89. doi: 10.1093/chemse/bjl030

Goldman, A. L., Van der Goes van Naters, W., Lessing, D., Warr, C. G., and Carlson, J. R. (2005). Coexpression of two functional odor receptors in one neuron. *Neuron* 45, 661–666. doi: 10.1016/j.neuron.2005.01.025

Gomez-Diaz, C., and Benton, R. (2013). The joy of sex pheromones. *EMBO Rep.* 14, 874–883. doi: 10.1038/embor.2013.140

Grewal, J. S., Nguyen, C., Robles, R., Cho, C., Kir, K., Fledderman, N., et al. (2014). Complex and non-redundant signals from individual odor receptors that underlie chemotaxis behavior in *Drosophila melanogaster* larvae. *Biol. Open* 3, 947–957. doi: 10.1242/bio.20148573

Grosjean, Y., Rytz, R., Farine, J.-P., Abuin, L., Cortot, J., Jefferis, G. S., et al. (2011). An olfactory receptor for food-derived odours promotes male courtship in Drosophila. *Nature* 478, 236–240. doi: 10.1038/nature10428

Guo, Y., Wang, Y., Wang, Q., and Wang, Z. (2014). The Role of PPK26 in Drosophila Larval Mechanical Nociception. *Cell Rep.* 9, 1183–1190. doi: 10.1016/j.celrep.2014.10.020

Ha, T. S., and Smith, D. P. (2006). A pheromone receptor mediates 11-cis-vaccenyl acetate-induced responses in Drosophila. *J. Neurosci.* 26, 8727–8733. doi: 10.1523/JNEUROSCI.0876-06.2006

Hallem, E. A., Ho, M. G., Carlson, J. R., and Haven, N. (2004). The molecular basis of odor coding in the Drosophila Antenna. *Cell* 117, 965–979. doi: 10.1016/j.cell.2004.05.012

Hallem, E. A., and Carlson, J. R. (2006). Coding of odors by a receptor repertoire. *Cell* 125, 143–160. doi: 10.1016/j.cell.2006.01.050

Hu, Y., Han, Y., Shao, Y., Wang, X., Ma, Y., Ling, E., et al. (2015). Gr33a modulates Drosophila male courtship preference. *Sci. Rep.* 5:7777. doi: 10.1038/srep07777

Idstein, H., Bauer, C., and Schreier, P. (1985). Volatile acids in tropical fruits: cherimoya (Annona cherimolia, Mill.), guava (psidium guajava, L.), mango (Mangifera indica, L., var. *Alphonso), papaya (Carica papaya, L.). Z. Lebensm. Unters. Forsch.* 180, 394–397. doi: 10.1007/BF01027773

Jallon, J. (1984). A few chemical words exchanged by Drosophila during courtship and mating. *Behav. Genet.* 14, 441–476. doi: 10.1007/BF01065444

Jiao, Y., Moon, S. J., and Montell, C. (2007). A Drosophila gustatory receptor required for the responses to sucrose, glucose, and maltose identified by mRNA tagging. *Proc. Natl. Acad. Sci. U.S.A.* 104, 14110–14115. doi: 10.1073/pnas.0702421104

Jiao, Y., Moon, S. J., Wang, X., Ren, Q., and Montell, C. (2008). Gr64f is required in combination with other gustatory receptors for sugar detection in Drosophila. *Curr. Biol.* 18, 1797–1801. doi: 10.1016/j.cub.2008.10.009

Jimenez-Del-Rio, M., Guzman-Martinez, C., and Velez-Pardo, C. (2010). The effects of polyphenols on survival and locomotor activity in *Drosophila melanogaster* exposed to iron and paraquat. *Neurochem. Res.* 35, 227–238. doi: 10.1007/s11064-009-0046-1

Jones, W. D., Cayirlioglu, P., Kadow, I. G., and Vosshall, L. B. (2007). Two chemosensory receptors together mediate carbon dioxide detection in Drosophila. *Nature* 445, 86–90. doi: 10.1038/nature05466

Joseph, R. M., Devineni, A. V., King, I. F. G., and Heberlein, U. (2009). Oviposition preference for and positional avoidance of acetic acid provide a model for competing behavioral drives in Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 106, 11352–11357. doi: 10.1073/pnas.0901419106

Khurana, S., and Siddiqi, O. (2013). Olfactory responses of Drosophila larvae. *Chem. Senses* 38, 315–323. doi: 10.1093/chemse/bjs144

Kim, S. H., Lee, Y., Akitake, B., Woodward, O. M., Guggino, W. B., Montell, C., et al. (2010). Drosophila TRPA1 channel mediates chemical avoidance in gustatory receptor neurons. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8440–8445. doi: 10.1073/pnas.1001425107

Koganezawa, M., Haba, D., Matsuo, T., and Yamamoto, D. (2010). The shaping of male courtship posture by lateralized gustatory inputs to male-specific interneurons. *Curr. Biol.* 20, 1–8. doi: 10.1016/j.cub.2009.11.038

Koh, T.-W., He, Z., Gorur-Shandilya, S., Menuz, K., Larter, N. K., Stewart, S., et al. (2014). The Drosophila IR20a clade of ionotropic receptors are candidate taste and pheromone receptors. *Neuron* 83, 850–865. doi: 10.1016/j.neuron.2014.07.012

Kohl, J., Ostrovsky, A. D., Frechter, S., and Jefferis, G. S. (2013). A bidirectional circuit switch reroutes pheromone signals in male and female brains. *Cell* 155, 1610–1623. doi: 10.1016/j.cell.2013.11.025

Kondoh, Y., Kaneshiro, K. Y., Kimura, K., and Yamamoto, D. (2003). Evolution of sexual dimorphism in the olfactory brain of Hawaiian Drosophila. *Proc. Biol. Sci.* 270, 1005–1013. doi: 10.1098/rspb.2003.2331

Kreher, S. A., Kwon, J. Y., and Carlson, J. R. (2005). The molecular basis of odor coding in the Drosophila larva. *Neuron* 46, 445–456. doi: 10.1016/j.neuron.2005.04.007

Kreher, S. A., Mathew, D., Kim, J., and Carlson, J. R. (2008). Translation of sensory input into behavioral output via an olfactory system. *Neuron* 59, 110–124. doi: 10.1016/j.neuron.2008.06.010

Kurtovic, A., Widmer, A., and Dickson, B. J. (2007). A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone. *Nature* 446, 542–546. doi: 10.1038/nature05672

Kwon, J. Y., Dahanukar, A., Weiss, L. A., and Carlson, J. R. (2007). The molecular basis of CO2 reception in Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3574–3578. doi: 10.1073/pnas.0700079104

Kwon, J. Y., Dahanukar, A., Weiss, L. A., and Carlson, J. R. (2011). Molecular and cellular organization of the taste system in the Drosophila larva. *J. Neurosci.* 31, 15300–15309. doi: 10.1523/JNEUROSCI.3363-11.2011

Kwon, Y., Kim, S. H., Ronderos, D. S., Lee, Y., Woodward, O. M., Guggino, W. B., et al. (2010). Drosophila TRPA1 channel is required to avoid the naturally occurring insect repellent citronellal. *Curr. Biol.* 20, 1672–1678. doi: 10.1016/j.cub.2010.08.016

Lacaille, F., Hiroi, M., Twele, R., Inoshita, T., Umemoto, D., Manière, G., et al. (2007). An inhibitory sex pheromone tastes bitter for Drosophila males. *PLoS ONE* 2:e661. doi: 10.1371/journal.pone.0000661

Laissue, P. P., and Vosshall, L. B. (2008). "The olfactory sensory map in Drosophila," in *Brain Development in Drosophila melanogaster*, ed G. M. Technau (Austin, TX: Landes Bioscience and Springer Science), 102–114. doi: 10.1007/978-0-387-78261-4_7

Larsson, M. C., Domingos, A. I., Jones, W. D., Chiappe, M. E., Amrein, H., Vosshall, L. B., et al. (2004). Or83b encodes a broadly expressed odorant receptor essential for Drosophila olfaction. *Neuron* 43, 703–714. doi: 10.1016/j.neuron.2004.08.019

Lebreton, S., Becher, P. G., Hansson, B. S., and Witzgall, P. (2012). Attraction of *Drosophila melanogaster* males to food-related and fly odours. *J. Insect Physiol.* 58, 125–129. doi: 10.1016/j.jinsphys.2011.10.009

Lee, K. P., Simpson, S. J., Clissold, F. J., Brooks, R., Ballard, J. W. O., Taylor, P. W., et al. (2008). Lifespan and reproduction in Drosophila: new insights from nutritional geometry. *Proc. Natl. Acad. Sci. U.S.A.* 105, 2498–2503. doi: 10.1073/pnas.0710787105

Lee, Y., Jun, S., and Montell, C. (2009). Multiple gustatory receptors required for the caffeine response in Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4495–4500. doi: 10.1073/pnas.0811744106

Lee, Y., Kang, M. J., Shim, J., Cheong, C. U., Moon, S. J., Montell, C., et al. (2012). Gustatory receptors required for avoiding the insecticide L-canavanine. *J. Neurosci.* 32, 1429–1435. doi: 10.1523/JNEUROSCI.4630-11.2012

Lee, Y., Kim, S. H., and Montell, C. (2010). Avoiding DEET through insect gustatory receptors. *Neuron* 67, 555–561. doi: 10.1016/j.neuron.2010.07.006

Liman, E. R., Zhang, Y. V., and Montell, C. (2014). Peripheral coding of taste. *Neuron* 81, 984–1000. doi: 10.1016/j.neuron.2014.02.022

Ling, F., Dahanukar, A., Weiss, L. A., Kwon, J. Y., and Carlson, J. R. (2014). The molecular and cellular basis of taste coding in the legs of Drosophila. *J. Neurosci.* 34, 7148–7164. doi: 10.1523/JNEUROSCI.0649-14.2014

Liu, L., Johnson, W. A., and Welsh, M. J. (2003a). Drosophila DEG/ENaC pickpocket genes are expressed in the tracheal system, where they may be involved in liquid clearance. *Proc. Natl. Acad. Sci. U.S.A.* 100, 2128–2133. doi: 10.1073/pnas.252785099

Liu, L., Leonard, A. S., Motto, D. G., Feller, M. A., Price, M. P., Johnson, W. A., et al. (2003b). Contribution of Drosophila DEG/ENaC genes to salt taste. *Neuron* 39, 133–146. doi: 10.1016/S0896-6273(03)00394-5

Liu, T., Starostina, E., Vijayan, V., and Pikielny, C. W. (2012). Two Drosophila DEG/ENaC channel subunits have distinct functions in gustatory neurons that activate male courtship. *J. Neurosci.* 32, 11879–11889. doi: 10.1523/JNEUROSCI.1376-12.2012

Liu, W., Liang, X., Gong, J., Yang, Z., Zhang, Y.-H., Zhang, J.-X., et al. (2011). Social regulation of aggression by pheromonal activation of Or65a olfactory neurons in Drosophila. *Nat. Neurosci.* 14, 896–902. doi: 10.1038/nn.2836

Lu, B., LaMora, A., Sun, Y., Welsh, M. J., and Ben-Shahar, Y. (2012). ppk23-Dependent chemosensory functions contribute to courtship behavior in *Drosophila melanogaster*. *PLoS Genet.* 8:e1002587. doi: 10.1371/journal.pgen.1002587

Marella, S., Fischler, W., Kong, P., Asgarian, S., Rueckert, E., and Scott, K. (2006). Imaging taste responses in the fly brain reveals a functional map of taste category and behavior. *Neuron* 49, 285–295. doi: 10.1016/j.neuron.2005.11.037

Markow, T. A. (1988). Reproductive Behavior of *Drosophila melanogaster* and D. *nigrospiracula* in the field and in the laboratory. *J. Comp. Psychol.* 102, 169–173. doi: 10.1037/0735-7036.102.2.169

Masek, P., and Keene, A. C. (2013). Drosophila fatty acid taste signals through the PLC pathway in sugar-sensing neurons. *PLoS Genet.* 9:e1003710. doi: 10.1371/journal.pgen.1003710

Mast, J. D., De Moraes, C. M., Alborn, H. T., Lavis, L. D., and Stern, D. L. (2014). Evolved differences in larval social behavior mediated by novel pheromones. *Elife* 3, 1–21. doi: 10.7554/eLife.04205

Mathew, D., Martelli, C., Kelley-Swift, E., Brusalis, C., Gershow, M., Samuel, A. D. T., et al. (2013). Functional diversity among sensory receptors in a Drosophila olfactory circuit. *Proc. Natl. Acad. Sci. U.S.A.* 110, E2134–E2143. doi: 10.1073/pnas.1306976110

McBride, C. S., Baier, F., Omondi, A. B., Spitzer, S. A., Lutomiah, J., Sang, R., et al. (2014). Evolution of mosquito preference for humans linked to an odorant receptor. *Nature* 515, 222–227. doi: 10.1038/nature13964

McMeniman, C. J., Corfas, R. A., Matthews, B. J., Ritchie, S. A., and Vosshall, L. B. (2014). Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. *Cell* 156, 1060–1071. doi: 10.1016/j.cell.2013.12.044

Meijerink, J., Braks, M. A., and Van Loon, J. J. (2001). Olfactory receptors on the antennae of the malaria mosquito Anopheles gambiae are sensitive to ammonia and other sweat-borne components. *J. Insect Physiol.* 47, 455–464. doi: 10.1016/S0022-1910(00)00136-0

Mellert, D. J., Robinett, C. C., and Baker, B. S. (2012). Doublesex functions early and late in gustatory sense organ development. *PLoS ONE* 7:e51489. doi: 10.1371/journal.pone.0051489

Min, S., Ai, M., Shin, S. A., and Suh, G. S. B. (2013). Dedicated olfactory neurons mediating attraction behavior to ammonia and amines in Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 110, E1321–E1329. doi: 10.1073/pnas.1215680110

Mishra, D., Miyamoto, T., Rezenom, Y. H., Broussard, A., Yavuz, A., Slone, J., et al. (2013). The molecular basis of sugar sensing in Drosophila larvae. *Curr. Biol.* 23, 1466–1471. doi: 10.1016/j.cub.2013.06.028

Mitri, C., Parmentier, M.-L., Pin, J.-P., Bockaert, J., and Grau, Y. (2004). Divergent evolution in metabotropic glutamate receptors. A new receptor activated by an endogenous ligand different from glutamate in insects. *J. Biol. Chem.* 279, 9313–9320. doi: 10.1074/jbc.M310878200

Mitri, C., Soustelle, L., Framery, B., Bockaert, J., Parmentier, M.-L., Grau, Y., et al. (2009). Plant insecticide L-canavanine repels Drosophila via the insect orphan GPCR DmX. *PLoS Biol.* 7:e1000147. doi: 10.1371/journal.pbio.1000147

Miyakawa, Y. (1981). Bimodal response in a chemotactic behaviour of Drosophila larvae to monovalent salts. *J. Insect Physiol.* 27, 387–392. doi: 10.1016/0022-1910(81)90016-0

Miyamoto, T., and Amrein, H. (2008). Suppression of male courtship by a Drosophila pheromone receptor. *Nat. Neurosci.* 11, 874–876. doi: 10.1038/nn.2161

Miyamoto, T., Slone, J., Song, X., and Amrein, H. (2012). A fructose receptor functions as a nutrient sensor in the Drosophila brain. *Cell* 151, 1113–1125. doi: 10.1016/j.cell.2012.10.024

Moat, A. G., Foster, J. W., and Spector, M. P. (eds.). (2002). "Fermentation pathways," in *Microbial Physiology* (Hoboken, NJ: Wiley-Liss), 412–433. doi: 10.1002/0471223867.ch11

Mollo, E., Fontana, A., Roussis, V., Polese, G., Amodeo, P., Ghiselin, M. T., et al. (2014). Sensing marine biomolecules: smell, taste, and the evolutionary transition from aquatic to terrestrial life. *Front. Chem.* 2:92. doi: 10.3389/fchem.2014.00092

Monte, P., Woodard, C., Ayer, R., Lilly, M., Sun, H., Carlson, J., et al. (1989). Characterization of the Larval Olfactory Response in Drosophila and Its Genetic Basis. *Behav. Genet.* 19, 267–283. doi: 10.1007/BF01065910

Montell, C. (2009). A taste of the Drosophila gustatory receptors. *Curr. Opin. Neurobiol.* 19, 345–353. doi: 10.1016/j.conb.2009.07.001

Moon, S. J., Köttgen, M., Jiao, Y., Xu, H., and Montell, C. (2006). A taste receptor required for the caffeine response *in vivo*. *Curr. Biol.* 16, 1812–1817. doi: 10.1016/j.cub.2006.07.024

Moon, S. J., Lee, Y., Jiao, Y., and Montell, C. (2009). A Drosophila gustatory receptor essential for aversive taste and inhibiting male-to-male courtship. *Curr. Biol.* 19, 1623–1627. doi: 10.1016/j.cub.2009.07.061

Neely, G. G., Keene, A. C., Duchek, P., Chang, E. C., Wang, Q.-P., Aksoy, Y. A., et al. (2011). TrpA1 regulates thermal nociception in Drosophila. *PLoS ONE* 6:e24343. doi: 10.1371/journal.pone.0024343

Neuhaus, E. M., Gisselmann, G., Zhang, W., Dooley, R., Störtkuhl, K., and Hatt, H., et al. (2005). Odorant receptor heterodimerization in the olfactory system of *Drosophila melanogaster*. *Nat. Neurosci.* 8, 15–17. doi: 10.1038/nn1371

Ng, S. H., Shankar, S., Shikichi, Y., Akasaka, K., Mori, K., Yew, J. Y., et al. (2014). Pheromone evolution and sexual behavior in Drosophila are shaped by male sensory exploitation of other males. *Proc. Natl. Acad. Sci. U.S.A.* 111, 3056–3061. doi: 10.1073/pnas.1313615111

Park, J.-H., and Kwon, J. Y. (2011). Heterogeneous expression of Drosophila gustatory receptors in enteroendocrine cells. *PLoS ONE* 6:e29022. doi: 10.1371/journal.pone.0029022

Parsons, P. A. (1980). Acetic acid vapour as a resource: threshold differences among three Drosophila species. *Experientia* 36, 1363. doi: 10.1007/BF01960099

Pikielny, C. W. (2012). Sexy DEG/ENaC channels involved in gustatory detection of fruit fly pheromones. *Sci. Signal.* 5:pe48. doi: 10.1126/scisignal.2003555

Possidente, D. R., and Murphey, R. K. (1989). Genetic control of sexually dimorphic axon morphology in Drosophila sensory neurons. *Dev. Biol.* 132, 448–457. doi: 10.1016/0012-1606(89)90241-8

Robertson, H. M., and Kent, L. B. (2009). Evolution of the gene lineage encoding the carbon dioxide receptor in insects. *J. Insect Sci.* 9, 19. doi: 10.1673/031.009.1901

Robertson, H. M., Warr, C. G., and Carlson, J. R. (2003). Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 100(Suppl.), 14537–14542. doi: 10.1073/pnas.2335847100

Rodrigues, V., and Hummel, T. (2008). "Development of the Drosophila olfactory system," in *Brain Development in Drosophila melanogaster*, ed G. M. Technau (Austin, TX: Landes Bioscience and Springer Science), 82–101. doi: 10.1007/978-0-387-78261-4_6

Ronderos, D. S., Lin, C.-C., Potter, C. J., and Smith, D. P. (2014). Farnesol-detecting olfactory neurons in Drosophila. *J. Neurosci.* 34, 3959–3968. doi: 10.1523/JNEUROSCI.4582-13.2014

Ruebenbauer, A., Schlyter, F., Hansson, B. S., Löfstedt, C., and Larsson, M. C. (2008). Genetic variability and robustness of host odor preference in *Drosophila melanogaster*. *Curr. Biol.* 18, 1438–1443. doi: 10.1016/j.cub.2008.08.062

Ruta, V., Datta, S. R., Vasconcelos, M. L., Freeland, J., Looger, L. L., Axel, R., et al. (2010). A dimorphic pheromone circuit in Drosophila from sensory input to descending output. *Nature* 468, 686–690. doi: 10.1038/nature09554

Schwartz, N. U., Zhong, L., Bellemer, A., and Tracey, W. D. (2012). Egg laying decisions in Drosophila are consistent with foraging costs of larval progeny. *PLoS ONE* 7:e37910. doi: 10.1371/journal.pone.0037910

Schwarz, S., Durisko, Z., and Dukas, R. (2014). Food selection in larval fruit flies: dynamics and effects on larval development. *Naturwissenschaften* 101, 61–68. doi: 10.1007/s00114-013-1129-z

Scott, K., Brady, R., Cravchik, A., Morozov, P., Rzhetsky, A., Zuker, C., et al. (2001). A chemosensory gene family encoding candidate gustatory and olfactory receptors in Drosophila. *Cell* 104, 661–673. doi: 10.1016/S0092-8674(01)00263-X

Silbering, A. F., and Galizia, C. G. (2007). Processing of odor mixtures in the Drosophila antennal lobe reveals both global inhibition

and glomerulus-specific interactions. *J. Neurosci.* 27, 11966–11977. doi: 10.1523/JNEUROSCI.3099-07.2007

Silbering, A. F., Rytz, R., Grosjean, Y., Abuin, L., Ramdya, P., Jefferis, G. S., et al. (2011). Complementary function and integrated wiring of the evolutionarily distinct Drosophila olfactory subsystems. *J. Neurosci.* 31, 13357–13375. doi: 10.1523/JNEUROSCI.2360-11.2011

Slone, J., Daniels, J., and Amrein, H. (2007). Sugar receptors in Drosophila Jesse. *Curr. Biol.* 17, 1809–1816. doi: 10.1016/j.cub.2007.09.027

Spehr, M., and Munger, S. D. (2009). Olfactory receptors: G protein-coupled receptors and beyond. *J. Neurochem.* 109, 1570–1583. doi: 10.1111/j.1471-4159.2009.06085.x

Starostina, E., Liu, T., Vijayan, V., Zheng, Z., Siwicki, K. K., Pikielny, C. W., et al. (2012). A Drosophila DEG/ENaC subunit functions specifically in gustatory neurons required for male courtship behavior. *J. Neurosci.* 32, 4665–4674. doi: 10.1523/JNEUROSCI.6178-11.2012

Stensmyr, M. C. (2003). Novel natural ligands for Drosophila olfactory receptor neurones. *J. Exp. Biol.* 206, 715–724. doi: 10.1242/jeb.00143

Stensmyr, M. C., Dekker, T., and Hansson, B. S. (2003). Evolution of the olfactory code in the *Drosophila melanogaster* subgroup. *Proc. Biol. Sci.* 270, 2333–2340. doi: 10.1098/rspb.2003.2512

Stensmyr, M. C., Dweck, H. K. M., Farhan, A., Ibba, I., Strutz, A., Mukunda, L., et al. (2012). A conserved dedicated olfactory circuit for detecting harmful microbes in Drosophila. *Cell* 151, 1345–1357. doi: 10.1016/j.cell.2012.09.046

Stocker, R. F. (1994). The organization of the chemosensory system in *Drosophila melanogaster*: a rewiew. *Cell Tissue Res.* 275, 3–26. doi: 10.1007/BF00305372

Stocker, R. F. (2008). "Design ofthe larval chemosensory system," in *Brain Development in Drosophila melanogaster*, ed G. M. Technau (Austin, TX: Landes Bioscience and Springer Science), 69–81. doi: 10.1007/978-0-387-78261-4_5

Stocker, R. F. (2009). The olfactory pathway of adult and larval Drosophila: conservation or adaptation to stage-specific needs? *Ann. N.Y. Acad. Sci.* 1170, 482–486. doi: 10.1111/j.1749-6632.2009.03896.x

Stockinger, P., Kvitsiani, D., Rotkopf, S., Tirián, L., and Dickson, B. J. (2005). Neural circuitry that governs Drosophila male courtship behavior. *Cell* 121, 795–807. doi: 10.1016/j.cell.2005.04.026

Suh, G. S. B., Wong, A. M., Hergarden, A. C., and Wang, J. W. (2004). A single population of olfactory sensory neurons mediates an innate avoidance behaviour in Drosophila. *Nature* 431, 854–858. doi: 10.1038/nature02980

Tennessen, J. M., and Thummel, C. S. (2011). Coordinating growth and maturation - insights from Drosophila. *Curr. Biol.* 21, R750–R757. doi: 10.1016/j.cub.2011.06.033

Thistle, R., Cameron, P., Ghorayshi, A., Dennison, L., and Scott, K. (2012). Contact chemoreceptors mediate male-male repulsion and male-female attraction during Drosophila courtship. *Cell* 149, 1140–1151. doi: 10.1016/j.cell.2012.03.045

Thorne, N., and Amrein, H. (2008). Atypical expression of Drosophila gustatory receptor genes in sensory. *J. Comp. Neurol.* 568, 548–568. doi: 10.1002/cne.21547

Thorne, N., Chromey, C., Bray, S., and Amrein, H. (2004). Taste perception and coding in drosophila. *Curr. Biol.* 14, 1065–1079. doi: 10.1016/j.cub.2004.05.019

Thum, A. S., Leisibach, B., Gendre, N., Selcho, M., and Stocker, R. F. (2011). Diversity, variability, and suboesophageal connectivity of antennal lobe neurons in D. melanogaster larvae. *J. Comp. Neurol.* 519, 3415–3432. doi: 10.1002/cne.22713

Toda, H., Zhao, X., and Dickson, B. J. (2012). The Drosophila female aphrodisiac pheromone activates ppk23(+) sensory neurons to elicit male courtship behavior. *Cell Rep.* 1, 599–607. doi: 10.1016/j.celrep.2012.05.007

Truman, J. W. (1990). Metamorphosis of the central nervous system of Drosophila. *J. Neurobiol.* 21, 1072–1084. doi: 10.1002/neu.480210711

Van der Goes van Naters, W., and Carlson, J. R. (2007). Receptors and neurons for fly odors in Drosophila. *Curr. Biol.* 17, 606–612. doi: 10.1016/j.cub.2007.02.043

Vijayan, V., Thistle, R., Liu, T., Starostina, E., and Pikielny, C. W. (2014). Drosophila pheromone-sensing neurons expressing the ppk25 ion channel subunit stimulate male courtship and female receptivity. *PLoS Genet.* 10:e1004238. doi: 10.1371/journal.pgen.1004238

Vijendravarma, R. K., Narasimha, S., and Kawecki, T. J. (2013). Predatory cannibalism in *Drosophila melanogaster* larvae. *Nat. Commun.* 4, 1789. doi: 10.1038/ncomms2744

Vosshall, L. B., Amrein, H., Morozov, P. S., Rzhetsky, A., and Axel, R. (1999). A Spatial Map of Olfactory Receptor Expression in the Drosophila Antenna. *Cell* 96, 725–736. doi: 10.1016/S0092-8674(00)80582-6

Wang, L., and Anderson, D. J. (2010). Identification of an aggression-promoting pheromone and its receptor neurons in Drosophila. *Nature* 463, 227–231. doi: 10.1038/nature08678

Wang, L., Han, X., Mehren, J., Hiroi, M., Billeter, J.-C., Miyamoto, T., et al. (2011). Hierarchical chemosensory regulation of male-male social interactions in Drosophila. *Nat. Neurosci.* 14, 757–762. doi: 10.1038/nn.2800

Wang, Z., Singhvi, A., Kong, P., and Scott, K. (2004). Taste representations in the Drosophila brain. *Cell* 117, 981–991. doi: 10.1016/j.cell.2004.06.011

Watanabe, K., Toba, G., Koganezawa, M., and Yamamoto, D. (2011). Gr39a, a highly diversified gustatory receptor in Drosophila, has a role in sexual behavior. *Behav. Genet.* 41, 746–753. doi: 10.1007/s10519-011-9461-6

Weiss, L. A., Dahanukar, A., Kwon, J. Y., Banerjee, D., and Carlson, J. R. (2011). The Molecular and cellular basis of bitter taste in Drosophila Linnea. *Neuron* 69, 258–272. doi: 10.1016/j.neuron.2011.01.001

Wisotsky, Z., Medina, A., Freeman, E., and Dahanukar, A. (2011). Evolutionary differences in food preference rely on Gr64e, a receptor for glycerol. *Nat. Neurosci.* 14, 1534–1541. doi: 10.1038/nn.2944

Xu, J., Sornborger, A. T., Lee, J. K., and Shen, P. (2008). Drosophila TRPA channel modulates sugar-stimulated neural excitation, avoidance and social response. *Nat. Neurosci.* 11, 676–682. doi: 10.1038/nn.2119

Yamamoto, D., and Koganezawa, M. (2013). Genes and circuits of courtship behaviour in Drosophila males. *Nat. Rev. Neurosci.* 14, 681–692. doi: 10.1038/nrn3567

Yanagawa, A., Guigue, A. M., and Marion-Poll, F. (2014). Hygienic grooming is induced by contact chemicals in *Drosophila melanogaster*. *Front. Behav. Neurosci.* 8:254. doi: 10.3389/fnbeh.2014.00254

Yang, C.-H., Belawat, P., Hafen, E., Jan, L. Y., and Jan, Y.-N. (2008). Drosophila egg-laying site selection as a system to study simple decision-making processes. *Science* 319, 1679–1683. doi: 10.1126/science.1151842

Yew, J. Y., Dreisewerd, K., Luftmann, H., Müthing, J., Pohlentz, G., Kravitz, E. A., et al. (2009). A new male sex pheromone and novel cuticular cues for chemical communication in Drosophila. *Curr. Biol.* 19, 1245–1254. doi: 10.1016/j.cub.2009.06.037

Zelle, K. M., Lu, B., Pyfrom, S. C., and Ben-Shahar, Y. (2013). The genetic architecture of degenerin/epithelial sodium channels in Drosophila. *G3 (Bethesda)* 3, 441–450. doi: 10.1534/g3.112.005272

Zhang, Y. V., Ni, J., and Montell, C. (2013a). The molecular basis for attractive salt-taste coding in Drosophila. *Science* 340, 1334–1338. doi: 10.1126/science.1234133

Zhang, Y. V., Raghuwanshi, R. P., Shen, W. L., and Montell, C. (2013b). Food experience – induced taste desensitization modulated by the Drosophila TRPL channel. *Nat. Neurosci.* 16, 1468–1476. doi: 10.1038/nn.3513

Zhou, C., Pan, Y., Robinett, C. C., Meissner, G. W., and Baker, B. S. (2014). Central brain neurons expressing doublesex regulate female receptivity in Drosophila. *Neuron* 83, 149–163. doi: 10.1016/j.neuron.2014.05.038

Ziegler, A. B., Berthelot-Grosjean, M., and Grosjean, Y. (2013). The smell of love in Drosophila. *Front. Physiol.* 4:72. doi: 10.3389/fphys.2013.00072

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Molecular basis of peripheral olfactory plasticity in *Rhodnius prolixus*, a Chagas disease vector

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Olfaction is fundamental for most animals and critical for different aspects of triatomine biology, including host-seeking, reproduction, avoidance of predators, and aggregation in shelters. Ethological and physiological aspects of these olfactory-mediated behaviors are well-understood, but their molecular bases are still largely unknown. Here we investigated changes in the molecular mechanisms at the peripheral olfactory level in response to different physiological and developmental conditions. For this, the antennal expression levels of the odorant (*Orco*) and ionotropic (*IR8a*, *IR25a*, and *IR76b*) coreceptor genes were determined in *Rhodnius prolixus* by means of quantitative real-time PCR (qRT-PCR) analysis. Gene expression changes were analyzed to test the effect of feeding and imaginal molt for both sexes. Moreover, we analyzed whether expression of these genes changed during the early life of adult bugs. Under these conditions bugs display distinct behavioral responses to diverse chemical stimuli. A significantly decreased expression was induced by blood feeding on all coreceptor genes. The expression of all genes was significantly increased following the imaginal molt. These results show that olfactory coreceptor genes have their expression altered as a response to physiological or developmental changes. Our study suggests that olfactory coreceptor genes confer adaptability to the peripheral olfactory function, probably underlying the known plasticity of triatomine olfactory-mediated behavior.

Keywords: olfaction, olfactory coreceptors, triatomines, behavior, physiology

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Introduction

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, is transmitted to humans and other mammals by hematophagous insects of the subfamily Triatominae (Reduviidae). This zoonosis is endemic to 22 countries in Central and South America, where 90–100 million people live in endemic areas, 8 million people are estimated to be infected, and 12,000–14,000 deaths are reported annually (Senior, 2007; Coura and Viñas, 2010; Rassi et al., 2010; Schmunis and Yadon, 2010). *Rhodnius prolixus* Stål, 1859 (Hemiptera: Reduviidae) is the second most important vector of Chagas disease, and the main species transmitting *T. cruzi* to humans in Colombia and Venezuela (Fitzpatrick et al., 2008; Guhl et al., 2009; Rassi et al., 2010; Hashimoto and Schofield, 2012). In the absence of a vaccine and an effective drug treatment, vector control is central to prevent the disease

(Rassi et al., 2010). Due to insecticide resistance in triatomine populations in Bolivia (Lardeux et al., 2010), and parts of Argentina and Venezuela (Vassena et al., 2000; González Audino et al., 2004), there is a dire need for the improvement or development of vector control strategies for sustained control of Chagas disease.

The olfactory system plays an essential role in several aspects of the biology of triatomines, such as shelter location (Lorenzo and Lazzari, 1996), food search (Núñez, 1982; Barrozo and Lazzari, 2004a,b), reproduction (Pontes et al., 2008; Vitta et al., 2009; Zacharias et al., 2010; Pontes and Lorenzo, 2012), and avoidance of predators (Ward, 1981; Manrique et al., 2006). In addition, *R. prolixus* locate hosts mainly through olfactory cues; hence their sense of smell directly regulates disease transmission (Guerenstein and Lazzari, 2009). An increased understanding of the olfactory system is therefore crucial for sustainable control of this disease vector. In other insects, considerable progress has been made in the understanding of the molecular basis of olfaction, which has fostered the development of novel olfactory-based strategies against agricultural pests and disease vectors (Kain et al., 2013; Tauxe et al., 2013). Two molecular components have been shown to be central for the detection of odorant stimuli in insects: the odorant receptors (ORs) (Clyne et al., 1999; Vosshall et al., 2000) and the ionotropic receptors (IRs) (Benton et al., 2009). Genes encoding for these proteins are expressed in olfactory sensory neurons (OSNs), primarily on the insect antennae (Carey and Carlson, 2011). ORs and IRs function as heteromeric odor-gated ion channels composed of one, or in the case of IRs up to five, variable subunits and one, or in the case of IRs up to three, obligate coreceptors: *Orco* (Vosshall et al., 2000; Larsson et al., 2004; Vosshall and Hansson, 2011), and *IR8a*, *IR25a* and *IR76b*, respectively (Benton et al., 2009; Abuin et al., 2011). These coreceptor proteins are also required for the trafficking of the heteromeric OR and IR complexes to the cilia of the OSNs (Larsson et al., 2004; Benton et al., 2006; Abuin et al., 2011).

Changes in the behavioral responsiveness to host signals and reproductive mates have been reported for bugs of this subfamily and correlated with the ingestion of a blood meal and adult maturation (Bodin et al., 2009b; Vitta and Lorenzo, 2009). Similar changes in vector behavior have been found to be correlated with alterations in gene expression in mosquitoes (Rinker et al., 2013; Omondi et al., 2015a). Regulation of gene transcription tentatively underlies the observed functional changes of the peripheral (Jang, 1995; Siju et al., 2010; Saveer et al., 2012; Omondi et al., 2015a) and central olfactory systems (Anton et al., 2007; Barrozo et al., 2011). The main objective of this report was to analyze ontogenetic and blood-meal induced changes in the transcript levels of OR and IR coreceptor genes in both sexes of *R. prolixus*. Based on behavioral observations we hypothesize that coreceptor gene expression is decreased in recently fed bugs. Moreover, we hypothesize that imaginal molting induces an increase in gene expression. We observed that *RproOrco, RproIR8a, RproIR25a,* and *RproIR76b* transcript levels are altered, in ways correlated with the significantly decreased behavioral responsiveness known for fed insects, as well as the acquisition of sexual signal detection capabilities in adults.

Materials and Methods

Insects

Experimental insects were obtained from the *R. prolixus* colony held at the Centro de Pesquisas René Rachou (CPqRR), which was established more than 20 years ago from a batch of domiciliary insects captured during field work in Honduras (donated by Dr. Carlos Ponce, Ministerio de Salud Pública, Honduras). Through the years, this colony has been kept as large as possible (ca. 20,000 insects) in order to preserve as much diversity as possible. Experimental insects were reared under controlled conditions at 26 ± 1°C, 65 ± 10% relative humidity, and at a 12 h:12 h light/dark cycle provided by artificial lights (4 fluorescent lamps, cold white light, 6400 K, 40 W). All experiments were performed with 5th instar larvae or adults, and all tests were developed separately for female and male insects. For experiments with immature insects, a group of 4th instar larvae of similar age was sorted and fed *ad libitum* with citrated rabbit blood (2.5% buffered sodium citrate, provided by Centro de Criação de Animais de Laboratório-CECAL, FIOCRUZ), using an artificial membrane feeder. After molting to the 5th instar, half of these insects were kept unfed, while the remaining bugs were offered blood *ad libitum* at day 16 after ecdysis. To obtain adult bugs for the remaining experiments, 5th instar larvae of similar age were sorted by sex and offered an *ad libitum* blood meal to induce their imaginal molt. As in the case of larvae, the feeding procedure was performed 16 days after the ecdysis of adult bugs. Transcript abundances for *RproOrco* and each IR coreceptor genes were analyzed separately for male and female bugs as follows: (i) unfed 21 day-old 5thinstar larvae; (ii) blood fed 21 day-old 5th instar larvae; (iii) unfed 1 day-old adult bugs; (iv) unfed 21 day-old adult bugs; and (v) blood fed 21 day-old adult bugs. All bug antennae were dissected between 10 am and 4 pm, and in the case of fed insects, antennae were cut 5 days after the ingestion of the blood meal. Each of the 5 treatments was replicated 6 times using pools of 60 antennae (i.e., 30 bugs) per sample.

Reference Genes and *R. prolixus* OR and IR Coreceptors

A set of candidate reference genes (**Table 1**) was selected because they were all previously used for qPCR normalization in triatomines (Majerowicz et al., 2011; Paim et al., 2012) and other insect species (Scharlaken et al., 2008; Lord et al., 2010; Ling and Salvaterra, 2011; Ponton et al., 2011). **Table 2** lists all reference factors calculated as the geometric means of the most stable combinations of these genes (Omondi et al., 2015b), used to evaluate changes in gene expression in the antennae of *R. prolixus*. The sequences of reference and target genes (*RproOrco*, *RproIR8a, RproIR25a,* and *RproIR76b*) were identified in the *R. prolixus* genome (available on www.vectorbase.org/organisms/rhodnius-prolixus) using a local tBLASTn algorithm (Altschul et al., 1997). Orthologous sequences were obtained from the Swiss Institute of Bioinformatics (Table S1 in Supplementary Material). Primers were designed using Primer3 4.0.0 (http://primer3.ut.ee/) (Rozen and Skaletsky, 2000) and compatibilities tested

TABLE 1 | Reference and target genes, biological function, primer sequences, amplicon and intron lengths, squared correlation coefficient, and qRT-PCR efficiency.

Gene	Biological function	Primer sequence (5′ to 3′)	Amplicon length (bp)	Intron length (bp)	R^2	*E* (%)
REFERENCE GENES						
Act	Cytoskeletal protein	For—TGTCTCCCACACTGTACCCATCTA/ Rev—TCGGTAAGATCACGACCAGCCAA	87	338	0.992	88.2%
eIF-1a	Protein biosynthesis	For—TTGGAGGCCATGTGCTTTGAT/ Rev—AGGTTTCTTGCTTCATCTGGAGT	94	183	0.999	91.3%
GAPDH	Glycolytic protein	For—GACTGGCATGGCATTCAGAGTT/ Rev—CCCCATTAAAGTCCGATGACACC	182	1130	0.992	102.5%
GST	Metabolism	For—TACCCATCATTTGGCGTGGACA/ Rev—CAAACCCAATTGCCTCAGCGAT	177	Intron—Exon junction	0.987	103.2%
G6PDH	Metabolism	For—AGCCTGGAGAAGCGGTTTACGTTA/ Rev—GTGAGCCACAGAATACGTCGAGT	162	923	0.998	96.5%
SDH	Metabolism	For—TTGCCGGAGTAGATGTTACCAG/ Rev—CAGCTGCATAAAGTCCTTCCAC	147	1592	0.999	104.8%
Sp	Metabolism	For—AGGGACCATCTTTGACTGCTCTTC/ Rev—GAATCACCCTGGCAAGCATCTTTT	157	Intron—Exon junction	0.996	98.8%
Tub	Structural subunit of microtubules	For—TGTGCCCAAGGATGTGAACG/ Rev—CACAGTGGGTGGTTGGTAGTTGAT	118	202	0.991	110.9%
TARGET GENES						
RproOrco	Odorant receptor coreceptor	For—GATCTGCACTGTTGCTGCAC/ Rev—CCATGGATGCAGAACACAAA	157	Intron—Exon junction	0.996	102.6%
RproIR8a	Ionotropic receptor coreceptor	For—TGCAGTCCAACAAGGTAGTCAC/ Rev—GCGTAATGCCTTCATCTTCGTCA	155	295	0.991	115.2%
RproIR25a	Ionotropic receptor coreceptor	For—AAGATGTGGCAGGCAATGAAGG/ Rev—CTGTTGCATCACCAAGGAAAGC	118	732	0.994	104.6%
RproIR76b	Ionotropic receptor coreceptor	For—GCGTTTGCGTACCAAATGGACA/ Rev—GCGTCCGGTAGATCCAAAGTGATT	113	1055	0.974	84.1%

R^2, squared correlation coefficient (calculated from the regression line of the standard curve); E, RT- qRT-PCR efficiency (calculated by the standard method).

TABLE 2 | Reference factors selected for each condition.

Condition	Normalizing factor for female antennae	Normalizing factor for male antennae
The effect of imaginal molt	*GST-Tub*	*SDH-GAPDH*
The effect of blood ingestion	*GST-Tub*	*SDH-GAPDH*
The effect of adult maturation	*GADPH-SDH*	All genes

with Oligoanalyser (Integrated DNA Technologies, Inc. IA, USA) softwares. The melting temperature was set at 60°C. The specificity for each primer was tested *in silico* using BLASTn (Altschul et al., 1990) in the *R. prolixus* genome database.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from pools of 60 antennae with 500 μL of TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, extracted RNA was resuspended in 30 μL of DEPC-treated water (Life Technologies), and its concentration was determined using a Qubit® 2.0 Fluorometer (Life Technologies). RNA integrity was analyzed by visualizing bands on agarose electrophoresis gels. Extraction of RNA was followed by a treatment using RQ1 RNase-Free DNase (Promega, Fitchburg, WI, USA). All treated RNA (11 μL per sample) was immediately used to synthesize cDNA using SuperScript III Reverse Transcriptase (Life Technologies) and a 1:1 mix of Random Hexamer and 10 μM Oligo$(dT)_{20}$ primers in a final volume of 20 μL.

Quantitative Real-time PCR

For quantitative real time PCR (qPCR) analysis, 10 μL of SYBR Green PCR Master Mix® (Life Technologies) were used in the reaction mixture that also contained 0.8 μL of a 10 μM primer solution and 1 μL of cDNA sample diluted two-fold in a final volume of 20 μL. The reactions were conducted using an ABIPRISM 7500 Sequence Detection System (Life Technologies) under the following conditions: one 10 min cycle at 95°C, followed by 40 cycles of 15 s at 95°C, 20 s at 60°C, and 30 s at 72°C. Following the amplification step, a melting curve analysis and an agarose gel electrophoresis were performed to confirm the specificity of the reaction. In all qPCR experiments, no-template controls (NTC) were included in triplicate for each primer set to verify the absence of exogenous DNA. For each experimental condition, six biological replicates were made, with three technical replicates performed for each of them. The PCR efficiencies (E) and repeatability (R^2) for each primer were determined using the slope of a linear regression model (Pfaffl, 2001). Information about primers, PCR amplicons and calibration curves is presented in **Table 1**. Besides, the output of

melt curve analysis for all primers is displayed in Figure S1 of Supplementary Material.

RT-PCR and Sequencing

Pure cDNA was used as a template for PCR reactions of the reference and target gene amplicons which were performed for 35 cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 30 s) with 2 μL of cDNA, 2.2 μL of a 1 mM dNTP solution, 1.2 μL of a 10 μM primer solution and 1 U of Taq polymerase (Promega) in a final volume of 20 μL. The size of the resulting PCR products was visualized by means of electrophoresis in agarose gels. These PCR products were purified using the Wizard Genomic DNA Purification Kit (Promega). The sequencing reactions for the purified products were performed with both primers using an ABI Prism BigDye V 3.1 Terminator Cycle Sequencing kit and an ABI 3730 DNA sequencing system (Life Technologies). The consensus sequences were obtained using the Staden Package 2.0 (Staden et al., 2000) and verified by comparing with the *R. prolixus* genomic database, using the basic local alignment search tool (BLASTn).

Gene Expression and Statistical Analysis

Data treatment for quantification cycle (*Cq*) values obtained from technical replicates followed standard procedures for qPCR (Livak and Schmittgen, 2001; Sengul and Tu, 2008). Briefly, readings from each set of technical replicates were checked for consistency using GenEx software (MultiD Analyses AB, Sweden) and then used to calculate mean *Cq*-values for each biological replicate. To determine the gene expression measures, the *Cq*-values were normalized to those of reference genes, and then to mean *Cq*-values obtained with a control treatment (Livak and Schmittgen, 2001; Sengul and Tu, 2008). By using such procedures, we have prioritized a data processing method that allows comparison to most qPCR analyses available in the literature. All raw *Cq*-values are presented in Table S2 of Supplementary Material.

The relative expression of *RproOrco*, *RproIR8a, RproIR25a,* and *RproIR76b* in female and male antennae was calculated in GenEx software (MultiD Analyses AB, Sweden) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). First, the expression levels of the four genes were normalized to the reference factors selected for each sex. Then, expression levels of each gene were normalized to the expression levels of unfed female and male larvae, respectively.

Fold-change values were subjected to statistical analysis to determine the effect of treatment on transcript abundance. All tests were performed separately for data obtained from female and male antennae and no comparisons were performed between sexes due to our experimental design. In order to inspect whether gene expression (y-var) was affected by developmental instar (larvae × adult) or feeding status (unfed × fed) (x-vars), data were subjected to Generalized Linear Modeling (GLM) under normal errors. Posterior residual analyses confirmed the choice of the error distribution and the suitability of the model. Modeling proceeded by building a full model, including all of the above parameters and their first order interactions and comparing this with a null model built without any of the above factors. In finding significant differences between null and full models, model simplification was performed on the latter by backward term extraction, removing one term at a time. Terms returned to the model if their removal provoked a change of deviance with $P < 0.05$. The minimum adequate model was defined as the one holding only significant terms. The procedure above was applied independently for male and female bugs for each of the genes under study (*RproOrco, RproIR8a, RproIR25a,* and *RproIR76b*), each new test using a distinct subset of data. All tests were performed using R version 3.2.0 (R Core Team, 2015).

Results

Results from statistical analyses are summarized in **Table 3** and **Figure 1**. Regardless of gender, the expression of all studied genes was enhanced in adults compared to larvae. Similarly, feeding depressed the expression of all coreceptor genes in larvae and adults, irrespective of their gender.

In males, developmental stage (i.e., larvae × adults) and feeding status (i.e., unfed × fed) acted independently from each other on the expression of all genes, except for *RproOrco*. This could be confirmed by looking at the non-significant interaction terms (stage:feeding) for the three ionotropic coreceptors (*RproIR8a*, *RproIR76b,* and *RproIR25a*), and the significant interaction term for *RproOrco* (**Table 3**). That is, unfed larvae differed from fed larvae in the same proportion as unfed adults differed from fed adults for the three ionotropic coreceptors (**Table 3**). For *RproOrco* expression, however, the effect of feeding was different from larvae and adults, as revealed by the significant interaction term for this specific case (**Table 3**).

As for females, there was a distinct pattern: an interdependence of developmental stage and feeding status affected the expression of all genes except for *RproIR76b*, as revealed by the interaction terms (stage:feeding) in **Table 3**. That is, the proportion by which unfed larvae differed from fed larvae was distinct from the proportion by which unfed adults differed from fed ones for *RproOrco*, *RproIR8a*, and *RproIR25a* (**Table 3**). Conversely, such proportions did not differ for *RproIR76b* (**Table 3**). In summary, the effects of developmental stage and feeding status tended to affect gene expression independently in males, the opposite occurring in females.

The results obtained with unfed 1-day-old adults are presented in Figure S2 in order to allow their comparison to those of unfed 21-day-old larvae and unfed 21-day-old adults.

Discussion

The abundance of antennal transcripts of the olfactory coreceptor genes of *R. prolixus* changes in response to development and blood feeding, and can also be affected by interactions between these factors. The results of this study reveal that the expression of olfactory coreceptor genes is a plastic process, closely linked to the observed changes in olfactory-mediated behaviors in these insects. Proper olfactory function requires the obligatory presence of coreceptors in a hypothetical fixed stoichiometry together with olfactory receptors (Vosshall et al., 2000; Benton et al., 2009; Abuin et al., 2011). This would

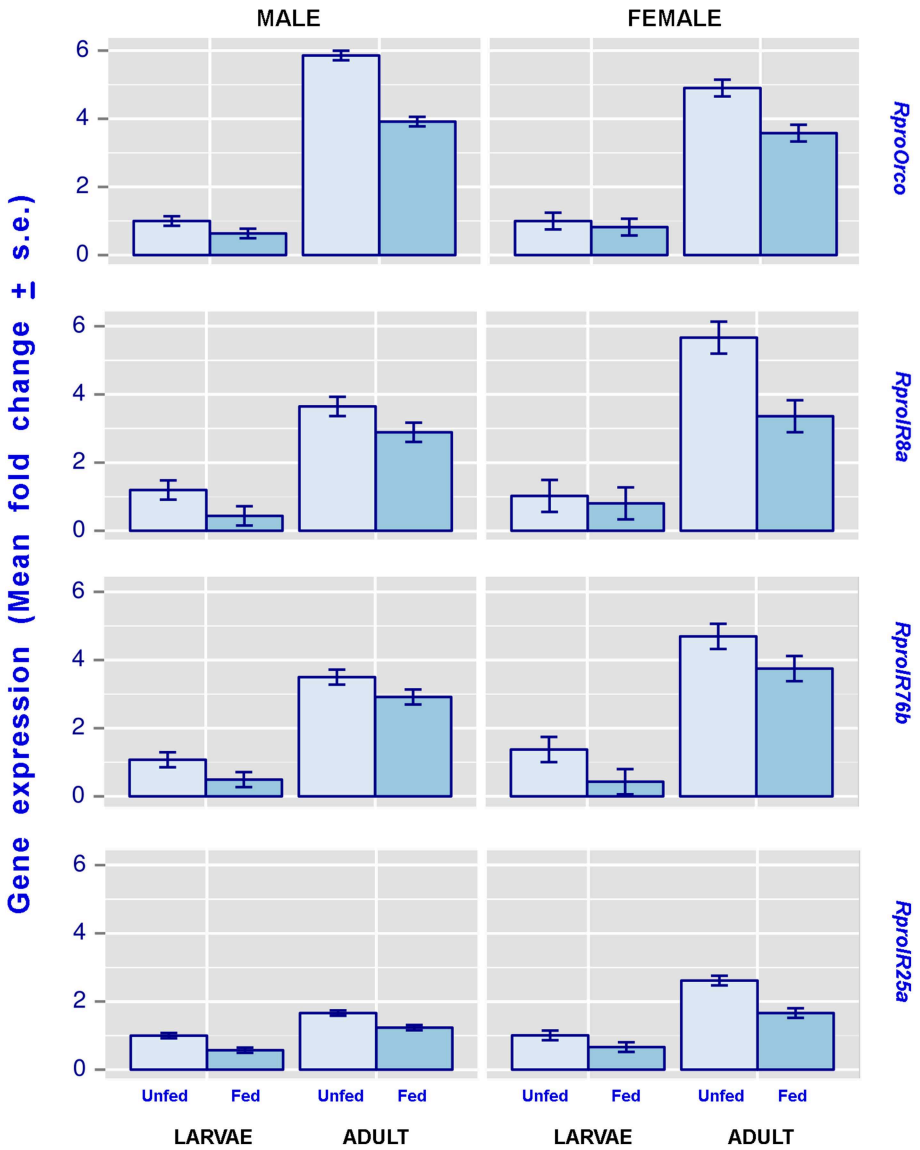

FIGURE 1 | The effect of imaginal molt and nutrition on male and female olfactory coreceptor gene expression levels, as seen in the antennae of 5th instar larvae and adults. Both 5th instar larvae and adults included in this figure were 21 day-old. Error bars represent the standard deviation generated from 6 replicates per condition. All statistical comparisons, i.e., the effect of the imaginal molt and the effect of feeding, were significantly different (more details about these comparisons can be observed in **Table 3**).

mean that alterations in coreceptor expression levels may reflect changes in the sensitivity of the olfactory system. Nonetheless, variations in coreceptor abundance may not reflect specific states of particular ORs or IRs. In fact, the changes in coreceptor expression reported here may have been the overall outcome of up or down regulation, or even absence of alteration, in specific receptors co-expressed with them. The expression of some olfactory receptors might be expected to remain unchanged in triatomines, e.g., those responsible for alarm or aggregation pheromone detection, as bugs are responsive to these stimuli irrespective of their nutritional or developmental state (Figueiras and Lazzari, 2000). Alternatively, other receptors related to functions that are dependent on good nutritional status may be anticipated to show an increase in expression, e.g., those that detect sexual pheromones (Vitta and Lorenzo, 2009).

The antennae of triatomines show a three-to-five-fold increase in the number of olfactory sensilla after their imaginal molt (Catalá, 1997; Gracco and Catalá, 2000; Akent'eva, 2008). Information about the ultrastructure of triatomines chemosensory sensilla is scarce, but available data suggest that triatomine trichoid sensilla may house up to 15 sensory neurons (Wigglesworth and Gillett, 1934). Rough estimates suggest that adult *R. prolixus* have approximately 1700 olfactory sensilla (Gracco and Catalá, 2000). Therefore, a concomitant increase in olfactory receptor expression would be expected when adult bug antennae are compared to those of fifth instar larvae.

TABLE 3 | Generalized Linear Modeling for olfactory coreceptor gene expression in male and female antennae of *Rhodnius prolixus*.

y-var	Model and Term	*d.f.*	*F*	*P*
MALE				
RproOrco	Full model	3;20	313.721	<0.001
	Stage	1;22	842.075	<0.001
	Feeding	1;21	67.549	<0.001
	Stage:Feeding	1;20	31.538	<0.001
RproIR8a	Full model	3;20	21.489	<0.001
	Stage	1;22	57.542	<0.001
	Feeding	1;21	5.514	<0.029
	Stage:Feeding	1;20	1.412	0.249
RproIR25a	Full model	3;20	24.408	<0.001
	Stage	1;22	51.691	<0.001
	Feeding	1;21	21.524	<0.001
	Stage:Feeding	1;20	0.01	0.922
RproIR76b	Full model	3;20	31.116	<0.001
	Stage	1;22	87.972	<0.001
	Feeding	1;21	5.079	<0.001
	Stage:Feeding	1;20	0.297	0.592
FEMALE				
RproOrco	Full model	3;20	66.202	<0.001
	Stage	1;22	183.831	<0.001
	Feeding	1;21	9.325	<0.001
	Stage:Feeding	1;20	5.449	0.03
RproIR8a	Full model	3;20	23.685	<0.001
	Stage	1;22	58.867	<0.001
	Feeding	1;21	7.237	0.014
	Stage:Feeding	1;20	4.95	0.038
RproIR25a	Full model	3;20	36.84	<0.001
	Stage	1;22	84.871	<0.001
	Feeding	1;21	21.001	<0.001
	Stage:Feeding	1;20	4.648	0.043
RproIR76b	Full model	3;20	25.096	<0.001
	Stage	1;22	66.74	<0.001
	Feeding	1;21	5.4	<0.001
	Stage:Feeding	1;20	3.148	0.091

Consistently our results showed that the antennal expression of all coreceptors studied presented a significant increase in 21-day-old adults (**Figure 1**). This indicates that both the OR and IR based olfactory subsystems (Silbering et al., 2011) seem to undergo a significant expansion in the adult phase of these hemimetabolous insects. Immature triatomines share several chemosensory mediated behaviors with adult bugs (Ward, 1981; Lorenzo Figueiras et al., 1994; Manrique et al., 2006; Guerenstein and Lazzari, 2009). These include the orientation to hosts, alarm and aggregation responses. Nevertheless, adult triatomines make use of sexual pheromones to find mates for reproduction (Pontes et al., 2008; Vitta et al., 2009; May-Concha et al., 2013) and the observed increase in coreceptor expression in adult antennae seems to support the hypothesis that a significant expansion is taking place on OR and IR subsystems to cope with sexual functions. Further experiments need to be performed to determine whether this proposal is indeed correct.

The increase in coreceptor expression observed for adults could be hypothesized to be originated either during the imaginal molt or at the initial phase of adult life. Newly molted bugs do not respond to cues associated with their vertebrate hosts and recently molted adults show a low behavioral responsiveness toward mates, unlike older ones (Bodin et al., 2009b; Vitta and Lorenzo, 2009). The latter happens despite the fact that the antennae of triatomines show an increase in the number of olfactory sensilla after the imaginal molt (Catalá, 1997; Gracco and Catalá, 2000; Akent'eva, 2008). Combined, previous behavioral studies and our gene expression analyses suggest that the peripheral olfactory system of *R. prolixus* undergoes a post-eclosion maturation process in adult bugs (Figure S2 in Supplementary Material). Similar maturation has been reported in female mosquitoes (Omondi et al., 2015a), which at early imaginal life do not express proper host-seeking behavior, have a decreased neural sensitivity to host volatiles and a lower expression level of olfactory receptor genes (Davis, 1984; Grant and O'Connell, 2007; Bohbot et al., 2013). Since proper olfactory function requires the obligatory presence of coreceptors (Vosshall et al., 2000; Benton et al., 2009; Abuin et al., 2011), alterations in coreceptor expression levels may induce changes in the sensitivity of the olfactory system of *R. prolixus,* ultimately leading to an increased behavioral responsiveness toward vertebrate host volatiles and pheromones in mature adults.

Larval *R. prolixus* display reduced electrophysiological responses to ammonia after ingesting a blood meal (Reisenman, 2014). Moreover, engorged triatomine larvae are refractory to host odor stimulation for a prolonged time after feeding (Bodin et al., 2009a) and remain hidden in shelters for several days while their molting is completed. A similar refractory period has been observed in blood fed mosquitoes (Klowden and Lea, 1979; Takken et al., 2001). In both *R. prolixus* (our study) and the mosquito *Anopheles gambiae* (Rinker et al., 2013), blood feeding induces a reduction in chemosensory gene transcript production. Moreover, both *A. gambiae* and *Aedes aegypti* mosquitoes have reduced electrophysiological responses to host odors during the refractory period post-blood meal (Takken et al., 2001; Siju et al., 2010). We suggest that coreceptor down-regulation would represent a way to shut down the system and save energy. It is interesting to note that the decrease induced by the blood meal tended to be more significant for adult bug antennae, when compared to larval expression. This was the case for *RproOrco* (in both sexes), *RproIr8a* and *RproIr25a* (only for females). Further experiments would be necessary to clarify the functional bases of the observed differences.

Our results show that changes in olfactory coreceptor gene transcripts seem to be linked with the observed plasticity in behavioral responsiveness of larval and adult *R. prolixus* to host volatiles and mates. How these changes are reflected in the functional characteristics of the peripheral and central olfactory systems requires further analysis. This report is the first in line for understanding the molecular basis of neurophysiological modulation of triatomine olfactory driven behaviors.

Author Contributions

JML, Provided most experimental data, analyzed data and wrote the manuscript; BO, Advised with experimental procedures, analyzed data, wrote, and provided comments on the manuscript; OD, Analyzed data and wrote the manuscript; IO, Provided experimental data; RI, Wrote and provided comments on the manuscript; ML, Conceived the project, help and advised on experiments, wrote, and provided comments on the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2015.00074

References

Abuin, L., Bargeton, B., Ulbrich, M., Isacoff, E., Kellenberger, S., and Benton, R. (2011). Functional architecture of olfactory ionotropic glutamate receptors. *Neuron* 69, 44–60. doi: 10.1016/j.neuron.2010.11.042

Akent'eva, N. A. (2008). The formation of the antenna sensory apparatus in some bug (Heteroptera) species in the course of their postembryonic development. *Entomol. Rev.* 88, 381–390. doi: 10.1134/s0013873808040015

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402. doi: 10.1093/nar/25.17.3389

Anton, S., Dufour, M. C., and Gadenne, C. (2007). Plasticity of olfactory guided behaviour and its neurobiological basis: lessons from moths and locusts. *Entomol. Exp. Appl.* 123, 1–11. doi: 10.1111/j.1570-7458.2007.00516.x

Barrozo, R. B., Jarriault, D., Deisig, N., Gemeno, C., Monsempes, C., Lucas, P., et al. (2011). Mating induced differential coding of plant odour and sex pheromone in a male moth. *Eur. J. Neurosci.* 33, 1841–1850. doi: 10.1111/j.1460-9568.2011.07678.x

Barrozo, R. B., and Lazzari, C. R. (2004a). The response of the blood-sucking bug *Triatoma infestans* to carbon dioxide and other host odours. *Chem. Senses* 29, 319. doi: 10.1093/chemse/bjh035

Barrozo, R. B., and Lazzari, C. R. (2004b). Orientation behaviour of the blood-sucking bug *Triatoma infestans* to short-chain fatty acids: synergistic effect of L-lactic acid and carbon dioxide. *Chem. Senses* 29, 833–841. doi: 10.1093/chemse/bjh249

Benton, R., Sachse, S., Michnick, S. W., and Vosshall, L. B. (2006). Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors *in vivo*. *PLoS Biol.* 4:e20. doi: 10.1371/journal.pbio.0040020

Benton, R., Vannice, K., Gomez-Diaz, C., and Vosshall, L. B. (2009). Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. *Cell* 136, 149–162. doi: 10.1016/j.cell.2008.12.001

Bodin, A., Vinauger, C., and Lazzari, C. R. (2009a). Behavioural and physiological state dependency of host seeking in the blood-sucking insect *Rhodnius prolixus*. *J. Exp. Biol.* 212, 2386–2393. doi: 10.1242/jeb.030668

Bodin, A., Vinauger, C., and Lazzari, C. R. (2009b). State-dependency of host-seeking in *Rhodnius prolixus*: the post-ecdysis time. *J. Insect Physiol.* 55, 574–579. doi: 10.1016/j.jinsphys.2009.02.004

Bohbot, J. D., Durand, N. F., Vinyard, B. T., and Dickens, J. C. (2013). Functional development of the octenol response in *Aedes aegypti*. *Front. Physiol.* 4:39. doi: 10.3389/fphys.2013.00039

Carey, A. F., and Carlson, J. R. (2011). Insect olfaction from model systems to disease control. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12987–12995. doi: 10.1073/pnas.1103472108

Catalá, S. S. (1997). Antennal sensilla of triatominae (Hemiptera, Reduviidae): a comparative study of five genera. *Int. J. Insect Morphol. Embryol.* 26, 67–73. doi: 10.1016/S0020-7322(97)00014-7

Clyne, P., Warr, C., Freeman, M., Lessing, D., Kim, J., and Carlson, J. R. (1999). A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron* 22, 327–338. doi: 10.1016/S0896-6273(00)81093-4

Coura, J. R., and Viñas, P. A. (2010). Chagas disease: a new worldwide challenge. *Nature* 465(7301), S6–S7. doi: 10.1038/nature09221

Davis, E. E. (1984). Development of lactic acid-receptor sensitivity and host-seeking behaviour in newly emerged female *Aedes aegypti* mosquitoes. *J. Insect Physiol.* 30, 211–215. doi: 10.1016/0022-1910(84)90005-2

Figueiras, A. N. L., and Lazzari, C. R. (2000). Temporal change of the aggregation response in *Triatoma infestans*. *Mem. Inst. Oswaldo Cruz* 95, 889–892. doi: 10.1590/S0074-02762000000600026

Fitzpatrick, S., Feliciangeli, M. D., Sanchez-Martin, M. J., Monteiro, F. A., and Miles, M. A. (2008). Molecular genetics reveal that silvatic *Rhodnius prolixus* do colonise rural houses. *PLoS Negl. Trop. Dis.* 2:e210. doi: 10.1371/journal.pntd.0000210

González Audino, P., Vassena, C., Barrios, S., Zerba, E., and Picollo, M. I. (2004). Role of enhanced detoxication in a deltamethrin-resistant population of *Triatoma infestans* (Hemiptera, Reduviidae) from Argentina. *Mem. Inst. Oswaldo Cruz* 99, 335–339. doi: 10.1590/S0074-02762004000300018

Gracco, M., and Catalá, S. S. (2000). Inter-specific and developmental differences on the array of antennal chemoreceptors in four species of Triatominae (Hemiptera: reduviidae). *Mem. Inst. Oswaldo Cruz* 95, 67–74. doi: 10.1590/S0074-02762000000100010

Grant, A. J., and O'Connell, R. J. (2007). Age-related changes in female mosquito carbon dioxide detection. *J. Med. Entomol.* 44, 617–623. doi: 10.1603/0022-2585(2007)44[617:ACIFMC]2.0.CO;2

Guerenstein, P. G., and Lazzari, C. R. (2009). Host-seeking: how triatomines acquire and make use of information to find blood. *Acta Trop.* 110, 148–158. doi: 10.1016/j.actatropica.2008.09.019

Guhl, F., Pinto, N., and Aguilera, G. (2009). Sylvatic triatominae: a new challenge in vector control transmission. *Mem. Inst. Oswaldo Cruz* 104 Suppl. 1, 71–75. doi: 10.1590/S0074-02762009000900012

Hashimoto, K., and Schofield, C. J. (2012). Elimination of *Rhodnius prolixus* in Central America. *Parasit. Vectors* 5:45. doi: 10.1186/1756-3305-5-45

Jang, E. B. (1995). Effects of mating and accessory gland injections on olfactory-mediated behavior in the female mediterranean fruit fly *Ceratitis capitata*. *J. Insect Physiol.* 41, 705–710. doi: 10.1016/0022-1910(95)00015-M

Kain, P., Boyle, S. M., Tharadra, S. K., Guda, T., Pham, C., Dahanukar, A., et al. (2013). Odour receptors and neurons for DEET and new insect repellents. *Nature* 502, 507–512. doi: 10.1038/nature12594

Klowden, M. J., and Lea, A. O. (1979). Humoral inhibition of host-seeking in *Aedes aegypti* during oocyte maturation. *J. Insect Physiol.* 25, 231–235. doi: 10.1016/0022-1910(79)90048-9

Lardeux, F., Depickère, S., Duchon, S., and Chavez, T. (2010). Insecticide resistance of *Triatoma infestans* (Hemiptera, Reduviidae) vector of Chagas disease in Bolivia. *Trop. Med. Int. Health* 15, 1037–1048. doi: 10.1111/j.1365-3156.2010.02573.x

Larsson, M. C., Domingos, A. I., Jones, W. D., Chiappe, M. E., Amrein, H., and Vosshall, L. B. (2004). Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* 43, 703–714. doi: 10.1016/j.neuron.2004.8.019

Ling, D., and Salvaterra, P. (2011). Robust RT-qPCR data normalization: validation and selection of internal reference genes during post-experimental data analysis. *PLoS ONE* 6:e17762. doi: 10.1371/journal.pone.0017762

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Lord, J., Hartzer, K., Toutges, M., and Oppert, B. (2010). Evaluation of quantitative PCR reference genes for gene expression studies in *Tribolium castaneum* after fungal challenge. *J. Microbiol. Methods* 80, 219–221. doi: 10.1016/j.mimet.2009.12.007

Lorenzo, M. G., and Lazzari, C. R. (1996). The spatial pattern of defecation in *Triatoma infestans* and the role of faeces as a chemical mark of the refuge. *J. Insect Physiol.* 42, 903–907. doi: 10.1016/0022-1910(96)00008-X

Lorenzo Figueiras, A. N., Kenigsten, A., and Lazzari, C. R. (1994). Aggregation in the haematophagous bug *Triatoma infestans*: chemical signals and temporal pattern. *J. Insect Physiol.* 40, 311–316. doi: 10.1016/0022-1910(94)90071-X

Tauxe, G. M., MacWilliam, D., Boyle, S. M., Guda, T., and Ray, A. (2013). Targeting a dual detector of skin and CO_2 to modify mosquito host seeking. *Cell* 155, 1365–1379. doi: 10.1016/j.cell.2013.11.013

Majerowicz, D., Alves-Bezerra, M., Logullo, R., Fonseca-De-Souza, A. L., Meyer-Fernandes, J. R., Braz, G. R., et al. (2011). Looking for reference genes for real-time quantitative PCR experiments in *Rhodnius prolixus* (Hemiptera: Reduviidae). *Insect Mol. Biol.* 20, 713–722. doi: 10.1111/j.1365-2583.2011.01101.x

Manrique, G., Vitta, A. C. R., Ferreira, R. A., Zani, C. L., Unelius, C. R., Lazzari, C. R., et al. (2006). Chemical communication in Chagas disease vectors. Source, identity, and potential function of volatiles released by the metasternal and brindley's glands of *Triatoma infestans* adults. *J. Chem. Ecol.* 32, 2035–2052. doi: 10.1007/s10886-006-9127-7

May-Concha, I., Rojas, J. C., Cruz-López, L., Millar, J. G., and Ramsey, J. M. (2013). Volatile compounds emitted by *Triatoma dimidiata*, a vector of Chagas disease: chemical analysis and behavioural evaluation. *Med. Vet. Entomol.* 27, 165–174. doi: 10.1111/j.1365-2915.2012.01056.x

Núñez, J. (1982). Food source orientation and activity in *Rhodnius prolixus* Stål (Hemiptera: Reduviidae). *Bull. Entomol. Res.* 72, 253–262. doi: 10.1017/S0007485300010555

Omondi, B. A., Latorre-Estivalis, J. M., Oliveira, I. H. R., Ignell, R., and Lorenzo, M. G. (2015b). Evaluation of reference genes for insect olfaction studies. *Parasit. Vectors* 8, 243. doi: 10.1186/s13071-015-0862-x

Omondi, B. A., Majeed, S., and Ignell, R. (2015a). Functional development of carbon dioxide detection in the maxillary palp of *Anopheles gambiae*. *J. Exp. Biol.* doi: 10.1242/jeb.116798. [Epub ahead of print].

Paim, R. M., Pereira, M. H., Di Ponzio, R., Rodrigues, J. O., Guarneri, A. A., Gontijo, N. F., et al. (2012). Validation of reference genes for expression analysis in the salivary gland and the intestine of *Rhodnius prolixus* (Hemiptera, Reduviidae) under different experimental conditions by quantitative real-time PCR. *BMC Res. Notes* 5:128. doi: 10.1186/1756-0500-5-128

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45. doi: 10.1093/nar/29.9.e45

Pontes, G. B., Bohman, B., Unelius, C., and Lorenzo, M. G. (2008). Metasternal gland volatiles and sexual communication in the triatomine bug, *Rhodnius prolixus*. *J. Chem. Ecol.* 34, 450–457. doi: 10.1007/s10886-008-9431-5

Pontes, G. B., and Lorenzo, M. G. (2012). Female metasternal gland odours mediate male aggregation in *Rhodnius prolixus*, a triatomid bug. *Med. Vet. Entomol.* 26, 33–36. doi: 10.1111/j.1365-2915.2011.00983.x

Ponton, F., Chapuis, M.-P., Pernice, M., Sword, G., and Simpson, S. (2011). Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in *Drosophila melanogaster*. *J. Insect Physiol.* 57, 840–850. doi: 10.1016/j.jinsphys.2011.03.014

Rassi, A. Jr., Rassi, A., and Marin-Neto, J. A. (2010). Chagas disease. *Lancet* 375, 1388–1402. doi: 10.1016/S0140-6736(10)60061-X

R Core Team. (2015). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Reisenman, C. E. (2014). Hunger is the best spice: effect of starvation in the antennal responses of the blood-sucking bug *Rhodnius prolixus*. *J. Insect Physiol.* 71, 8–13. doi: 10.1016/j.jinsphys.2014.09.009

Rinker, D. C., Pitts, R. J., Zhou, X., Suh, E., Rokas, A., and Zwiebel, L. J. (2013). Blood meal-induced changes to antennal transcriptome profiles reveal shifts in odor sensitivities in *Anopheles gambiae*. *Proc. Natl. Acad. Sci. U.S.A.* 110, 8260–8265. doi: 10.1073/pnas.1302562110

Rozen, S., and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* 132, 365–386. doi: 10.1385/1-59259-192-2:365

Saveer, A. M., Kromann, S. H., Birgersson, G., Bengtsson, M., Lindblom, T., Balkenius, A., et al. (2012). Floral to green: mating switches moth olfactory coding and preference. *Proc. Biol. Sci.* 279, 2314–2322. doi: 10.1098/rspb.2011.2710

Scharlaken, B., de Graaf, D. C., Goossens, K., Brunain, M., Peelman, L. J., and Jacobs, F. J. (2008). Reference gene selection for insect expression studies using quantitative real-time PCR: the honeybee, Apis mellifera, head after a bacterial challenge. *J. Insect Sci.* 8:33. doi: 10.1673/031.008.3301

Schmunis, G. A., and Yadon, Z. E. (2010). Chagas disease: a Latin American health problem becoming a world health problem. *Acta Trop.* 115, 14–21. doi: 10.1016/j.actatropica.2009.11.003

Sengul, M. S., and Tu, Z. (2008). Characterization and expression of the odorant-binding protein 7 gene in *Anopheles stephensi* and comparative analysis among five mosquito species. *Insect Mol. Biol.* 17, 631–645. doi: 10.1111/j.1365-2583.2008.00837

Senior, K. (2007). Chagas disease: moving towards global elimination. *Lancet Infect. Dis.* 7, 572. doi: 10.1016/S1473-3099(07)70194-9

Siju, K., Hill, S. R., Hansson, B. S., and Ignell, R. (2010). Influence of blood meal on the responsiveness of olfactory receptor neurons in antennal sensilla trichodea of the yellow fever mosquito *Aedes aegypti*. *J. Insect Physiol.* 56, 659–665. doi: 10.1016/j.jinsphys.2010.02.002

Silbering, A., Rytz, R., Grosjean, Y., Abuin, L., Ramdya, P., Jefferis, G., et al. (2011). Complementary function and integrated wiring of the evolutionarily distinct *Drosophila* olfactory subsystems. *J. Neurosci.* 31, 13357–13375. doi: 10.1523/JNEUROSCI.2360-11.2011

Staden, R., Beal, K. F., and Bonfield, J. K. (2000). The Staden package, 1998. *Methods Mol. Biol.* 132, 115–130. doi: 10.1385/1-59259-192-2:115

Takken, W., Van Loon, J. J., and Adam, W. (2001). Inhibition of host-seeking response and olfactory responsiveness in *Anopheles gambiae* following blood feeding. *J. Insect Physiol.* 47, 303–310. doi: 10.1016/S0022-1910(00)00107-4

Vassena, C. V., Picollo, M. I., and Zerba, E. N. (2000). Insecticide resistance in Brazilian *Triatoma infestans* and Venezuelan *Rhodnius prolixus*. *Med. Vet. Entomol.* 14, 51–55. doi: 10.1046/j.1365-2915.2000.00203.x

Vitta, A. C., and Lorenzo, M. G. (2009). Copulation and mate guarding behavior in *Triatoma brasiliensis* (Hemiptera: Reduviidae). *J. Med. Entomol.* 46, 789–795. doi: 10.1603/033.046.0409

Vitta, A. C. R., Bohman, B., Unelius, C. R., and Lorenzo, M. G. (2009). Behavioral and electrophysiological responses of *Triatoma brasiliensis* males to volatiles produced in the metasternal glands of females. *J. Chem. Ecol.* 35, 1212–1221. doi: 10.1007/s10886-009-9709-2

Vosshall, L. B., and Hansson, B. S. (2011). A unified nomenclature system for the insect olfactory coreceptor. *Chem. Senses* 36, 497–498. doi: 10.1093/chemse/bjr022

Vosshall, L. B., Wong, A. M., and Axel, R. (2000). An olfactory sensory map in the fly brain. *Cell* 102, 147–159. doi: 10.1016/S0092-8674(00)00021-0

Ward, J. P. (1981). A comparison of the behavioural responses of the haematophagous bug, *Triatoma infestans*, to synthetic homologues of two naturally occurring chemicals (n- and iso-butyric acid). *Physiol. Entomol.* 6, 325–329. doi: 10.1111/j.1365-3032.1981.tb00277.x

Wigglesworth, V. B., and Gillett, J. D. (1934). The function of the antennae in *Rhodnius prolixus* (Hemiptera) and the mechanism of orientation to the host. *J. Exp. Biol.* 11, 120–139.

Zacharias, C. A., Pontes, G. B., Lorenzo, M. G., and Manrique, G. (2010). Flight initiation by male *Rhodnius prolixus* is promoted by female odors. *J. Chem. Ecol.* 36, 449–451. doi: 10.1007/s10886-010-9779-1

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The narrowing olfactory landscape of insect odorant receptors

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The molecular basis of odorant detection and its corollary, the task of the odorant receptor, are fundamental to understanding olfactory coding and sensory ecology. Based on their molecular receptive range, olfactory receptors have been classified as pheromone and non-pheromone receptors, which are respectively activated by a single pheromone component ("specialist") or by multiple odorant ligands ("generalist"). This functional distinction is unique among ligand-gated ion channels and has shaped how we model olfactory coding both at the peripheral and central levels. Here, we revisit the long-standing combinatorial theory of olfaction and argue, based on physiological, pharmacological, evolutionary, and experimental grounds that the task of the odorant receptor is not different from that of neurotransmitter receptors localized in neuronal synapses.

Keywords: odorant receptor, ligand-gated ion channel, synaptic communication, olfactory communication, neurotransmitter receptors

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Introduction

How insects process odorants is a central question in the field of olfactory neurobiology. The number of green leaf volatiles (GLVs) produced by plants and volatile organic compounds (VOCs) emitted by animals or other sources (rotting fruits and excretion products) are not well-defined. Most recent studies report that GLVs (Dudareva et al., 2006; Knudsen et al., 2006) and VOCs number just below 2000 chemicals (Penn et al., 2007; Phillips et al., 2013; de Lacy Costello et al., 2014) (**Figure 1A**), which is likely an underestimate. Insects rely on odorant receptor (*Or*), ionotropic receptor (*Ir*), and the CO_2-sensing gustatory receptor (*Gr*) gene families for the long-range detection of airborne chemical cues (Suh et al., 2014). Until now, the *Or* clade has been the most extensively studied from both evolutionary and functional standpoints, and will be the focus of this theory article.

Odorant Receptors (ORs) are at the front line of odorant detection and much like neurotransmitter receptors (NRs), their task is to convert chemical signals into electrical outputs (**Figure 1B**) thereby ensuring the continuity of information flowing from the environment to the brain. Functional ORs are heteromeric complexes composed of an odorant-sensing unit belonging to a large and diverse family and a conserved *OR co*-receptor named ORco (for review, see Suh et al., 2014). These OR complexes are localized in the dendrites of olfactory receptor neurons (ORNs) embedded in sensilla, which project from the insect cuticular surface on olfactory appendages.

Insect OR repertoires vary greatly in number, ranging from 0 in the bristletail (Missbach et al., 2014), 110 in the yellow fever mosquito *Aedes aegypti* (Bohbot et al., 2007), 170 in the honeybee *Apis mellifera* (Robertson and Wanner, 2006) to over 400 ORs in eusocial ants (Zhou et al., 2012). Despite such a limited number of ORs, insects navigate complex chemical environments by

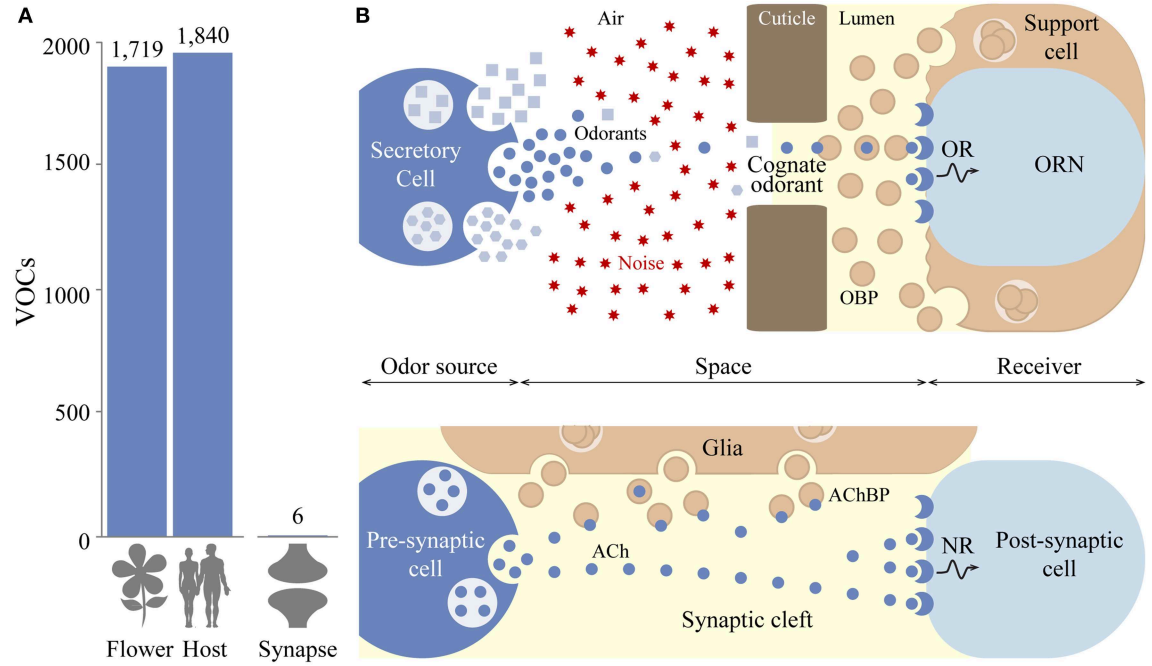

FIGURE 1 | Ecological context of olfactory and synaptic communications. (A) Human and plant volatile organic compounds (VOCs) number in the thousands, of which an unknown number of odorants are involved in animal–insect and plant–insect interactions. By contrast, few neurotransmitters and co-transmitters are present in the synaptic cleft. **(B)** Synaptic and olfactory communications are tripartite systems: large quantities of chemicals are secreted by an odor source (e.g., a secretory cell or organic matter), come into contact with binding proteins secreted by glia or support cells and activate receptors on the surface of receiver cells, which convert these chemical signals into electrical outputs. The main difference between olfactory and synaptic communications is that the concentration of cognate ligands that reach neurotransmitter receptors (NRs) and odorant receptors (ORs) is hypothesized to be orders of magnitude different. In cholinergic synapses, glia release acetylcholine-binding protein (AChBP) to capture acetylcholine (Ach) and suppress synaptic transmission. The hydrophobic nature of odorants may suffice to keep the sensillum lymph relatively free of chemical noise while odorant-binding proteins (OBPs) capture ecologically relevant odorant to foster OR activation.

exhibiting remarkable olfactory sensitivity, considering that some of these airborne VOCs are present in the air in the picomolar range (Phillips, 1997). The impact of this information on insect fitness, both in terms of survival and reproduction cannot be overstated. Host species must be located, conspecifics recognized, trails followed, and potential dangers avoided during many stages of the insect life cycle. Thus, it is tempting to surmise that ORs have evolved high sensitivity and selectivity capabilities for the detection of ecologically meaningful odorants.

The combinatorial theory of olfaction (Malnic et al., 1999) explains how this limited set of "generalist" ORs, thereafter referred to as non-pheromone receptors (nPRs), encode thousands of odorants. This weak shape theory (Rinaldi, 2007), a "relaxed" version of the stereochemical model of olfaction (Moncrieff, 1949; Amoore, 1963), postulates that an nPR only recognizes a part of the odorant and therefore can accommodate a variety of odorants provided they share common chemical features. Functional studies on *Drosophila melanogaster* (Hallem et al., 2004) and *Anopheles gambiae* (Carey et al., 2010; Wang et al., 2010) support the theory that nPRs exhibit broad molecular receptivity. However, moth pheromone receptors (PRs) function according to a more rigid lock-and-key mechanism as they are activated by single pheromone components (Große-Wilde et al., 2007; Miura et al., 2009; Wang et al., 2011; Leary et al., 2012; Sun et al., 2013). Together, nPRs and PRs allow the peripheral olfactory system to encode the identity and quantity of odorants over a wide range of molecules and concentrations. This model continues to have important implications on the conceptual roles of the peripheral and central nervous systems in terms of signal filtering and olfactory coding, respectively.

In recent years, the discovery of an increasing number of specialized nPRs in moths and mosquitoes has broken the pharmacological monopoly of PRs in terms of sensitivity and specificity. This recent development is cause for revisiting the concepts of molecular receptive range and olfactory tuning as well as their respective contributions to the theory of combinatorial receptor codes for odorants in insects. This article continues an earlier discussion on the task of the OR (Bohbot and Dickens, 2012a). Here, we have compared the physiological contexts and pharmacological properties of ORs and NRs by discussing the notions of receptive range and olfactory tuning from the perspective of chemical ecology and evolution and review the experimental designs supporting the notion of generalist ORs, which ultimately have shaped the combinatorial theory of olfaction. Finally, we comment on the respective roles of the peripheral and central nervous systems in olfactory coding and propose strategies for testing our ideas.

Olfactory and Synaptic Communications

The foundation of our discussion begins with a deceptively simple question: is the task of an insect OR different from that of a NR (Firestein, 2001)? Although not the focus of this article, ionotropic glutamate receptors provide a clear example of an evolutionarily ancient mechanism linking olfactory and synaptic communications (Croset et al., 2010). In both cases, a chemical signal travels through space to interact with binding-proteins and degrading enzymes (Vogt and Riddiford, 1981; Smit et al., 2001), which modulate the signal before being detected by an ionotropic receptor (**Figure 1B**). Despite these similarities, the chemical constraints on ORs and NRs differ in one fundamental way. In the case of synaptic neurotransmission, millimolar concentrations of water-soluble neurotransmitters are released in the synaptic cleft (Kuffler and Yoshikami, 1975; Clements, 1996; Karayannis et al., 2010), a space several nanometers wide (Stocker and Nuesch, 1975; Felten and Olschowka, 1987) whose chemical content is regulated by the local cellular environment. Synaptic clefts may contain one or more neurotransmitters and co-transmitters (**Figure 1B**), but their exact compositions remain largely unknown (Burnstock, 2004). Considering that these conditions are optimal for synaptic transmission, it may be sufficient that NRs act as low-sensitivity receptors (millimolar range) much like the *Bombyx mori* GR9, a highly selective sugar receptor activated by millimolar concentrations of D-fructose that functions as an ionotropic receptor (Sato et al., 2011) (**Figure 2A**). Moreover, low-sensitivity in the synapse reduces potential noise associated with spontaneous neurotransmitter release (Faisal et al., 2008).

By comparison, ORs potentially face a far more complex chemical environment. With greater distances form the emitting sources and due to the chaotic nature of turbulent air, the occurrence of any particular cognate odorant reaching an OR is orders of magnitude lower than the probability of neurotransmitters interacting with NRs. The presence of odorant molecules competing for the recognition sites of ORs is a matter of conjecture but it is likely that due to their hydrophobic nature, most odorants do not cross the sensillum lymph barrier unless helped by transporter proteins such as odorant-binding proteins (**Figure 1B**). Provided that these odorant-binding proteins are selective, the sensillum lymph and synaptic cleft may therefore be similar in terms of chemical complexity. To increase selective OR-odorant binding probability events, insects have evolved several anatomical and biochemical adaptations including elongated porous sensillae and binding proteins to facilitate the transport of cognate odorants through the sensillum lymph. Based on these parameters, we suggest that ORs are likely to be more sensitive and equally specific toward their cognate ligands than NRs.

Comparative Pharmacology of ORs and NRs

How do ligand-receptor interactions differ in terms of sensitivity and specificity in the context of olfactory and synaptic communications? To address this question, we have compared these pharmacological features between ORs and other ligand-gated ion channels (LGICs) including cys-loop receptors, ionotropic glutamate receptors, and ATP-gated channels. We surveyed the scientific literature and compared 50 data points representing the EC_{50}-values (concentration of ligand that elicits 50% of maximum receptor activation) of NRs, nPRs, PRs, and one sugar GR (Supplementary Table 1) expressed in *Xenopus laevis* oocytes (**Figure 2A**). This expression system provided the largest EC_{50} dataset of LGICs.

NRs exhibit sensitivities in the nanomolar and micromolar range. Nicotinic acetylcholine receptors (nAChRs) have the lowest and broadest range of sensitivities to acetylcholine, which is likely caused by the use of non-native subunit combinations (Chavez-Noriega et al., 1997; Wonnacott and Barik, 2007). Ligands for the serotonin, glutamate, GABA, and N-methyl-D-aspartate receptors display relatively narrower potencies in the nanomolar range (**Figure 2A**), which, contrary to our assumption, shows that LGICs are highly sensitive to their cognate ligand.

The majority of PRs also exhibit EC_{50}-values in the nanomolar range with the moths *Ostrinia furnicalis* OR3 showing the highest sensitivity to (Z)-11-tetradecenyl acetate (Leary et al., 2012) and *Heliothis armigera* OR13 showing the lowest sensitivity to (Z)-11-hexadecenal (Liu et al., 2013) (**Figure 2A**). All the nPRs for which cognate ligands have been identified using sensory physiology and behavior exhibit EC_{50}-values in the nanomolar range as well, suggesting that the distinction between PRs and non-PRs on the bases of sensitivity is unwarranted provided that the cognate ligands are used. Recently, a "generalist" OR has been shown to detect the GLV E-β-farnesene in the nanomolar range demonstrating at the molecular level that GLV-sensing ORs can be highly sensitive and specific (Liu et al., 2014).

Receptor specificity is the ability to distinguish between a cognate odorant ligand and its closest structural analog. We used EC_{50}-values to benchmark the discriminative power of PRs and nPRs, an aspect of LGIC pharmacology that has been scarcely studied, which limits our ability to draw general conclusions. Nonetheless, we find examples of ORs that display clear differential sensitivities ranging from 1 to 2 orders of magnitude (**Figure 2B**), suggesting that these receptors have evolved high stereospecificity, which likely reflect their ecological relevance (Bohbot and Dickens, 2009). Several mosquito ORs show superior recognition abilities (Hughes et al., 2010; Pelletier et al., 2010) compared to some moth PRs, which discriminate between geometric pheromone isomers (Wanner et al., 2010; Leary et al., 2012). Considering how little to no recognition PRs and nPRs display toward structural analogs of the cognate odorant, it is interesting to note that some PRs are more robustly activated by the formate analogs of known aldehyde constituents of moth pheromones (Xu et al., 2012). This observation might suggest that "super" ligands may be discovered by exploring close structural analogs of known cognate ligands. However, the selectivity of the octenol receptor (OR8) from *A. aegypti* suggests that any modifications to the cognate ligands elicit little to no receptor activation (Bohbot and Dickens, 2009). Whatever the case may be, detailed studies of formate pheromone derivatives will help clarify our understanding of receptor-ligand molecular relationships and may lead to the development of novel

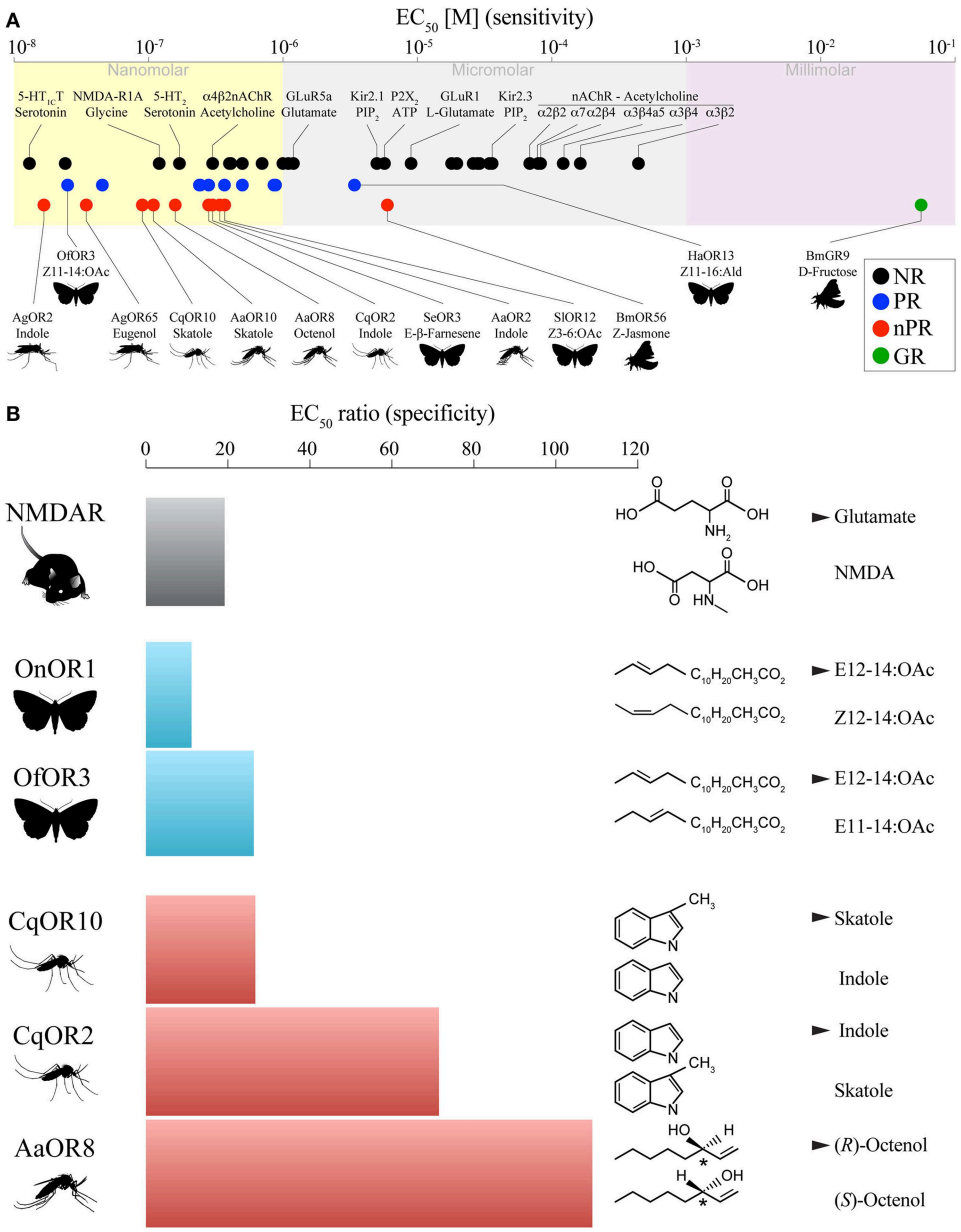

FIGURE 2 | Comparative pharmacology of ligand-gated ion channels and odorant receptors. (A) The sensitivity of a receptor toward its natural ligand is characterized by the Effective Concentration 50 (EC_{50}), which is the concentration eliciting 50% of the receptor maximal response. Except for many acetylcholine receptors and the gustatory receptor 9 (GR9), the sensitivities of neurotransmitter receptors (NRs), pheromone receptors (PRs) and non-pheromone receptors (nPRs) are in the nanomolar range. **(B)** Receptor specificity (EC_{50} ratio) is a measure of receptor preference between a cognate ligand and a related structural analog. For instance, NMDAR requires 19 times more NMDA to reach the activation level elicited by glutamate. Some nPRs exhibit higher specificity than some PRs and the N-methyl-D-aspartate receptor (NMDAR). Cognate ligands are indicated by a solid triangle. References and species name abbreviations can be found in Supplementary Table 1.

bioactive molecules affecting the behavior of agricultural and medical pests.

ORs as Ecological Adaptations

The combinatorial receptor coding scheme posits that the majority of ORs, with the exclusion of PRs, are promiscuous in terms of odorant recognition. While it provides an attractive model to encode a wide variety of odorant cues, it is counterintuitive from an evolutionary standpoint. Such an olfactory system would potentially be exposed to continuous overstimulation and thus lack the ability to distinguish important signals from background noise. In addition, the central nervous system would be required to filter this information into a useful code. What evolutionary

mechanism would explain how olfactory systems develop such a state?

Distinctive features of insect ORs include high evolutionary rates (Neafsey et al., 2015), lineage-specific expansions (Hill et al., 2002; Robertson and Wanner, 2006; Zhou et al., 2012; Cande et al., 2013) and large variations in gene repertoires (Nei et al., 2008; Sánchez-Gracia et al., 2009). The major mechanism of evolution of the *Or* gene family follows the birth-and-death model (Sánchez-Gracia et al., 2009) whereby genes multiply via tandem gene duplication events and are removed by deletion (Gardiner et al., 2008). Studies on *Or* evolution in *Drosophila* (McBride, 2007; McBride et al., 2007; Gardiner et al., 2008; Sánchez-Gracia et al., 2009; Stensmyr et al., 2012) and *Anopheles* mosquitoes (Neafsey et al., 2015) indicate that the birth-and-death of *Or* genes is not random (McBride et al., 2007) but that genetic diversity and variability are principally acted upon by purifying selection. Despite these observations, *Or* genes exhibit some of the highest level of positive selection in many lineage specific expansions suggesting functional divergence associated with host specialization (McBride et al., 2007; Neafsey et al., 2015), and mate selection (Leary et al., 2012).

The mosquito-specific indole receptors show remarkable sequence conservation across the Culicinae and Anophelinae families (Bohbot et al., 2007), indicating that they fulfill fundamental olfactory functions critical to the life cycle of these insects. These receptors diversified through several instances of duplication events followed by positive selection, which diversified their tuning range toward indole analogs, functions that have subsequently been maintained by purifying selection. It appears that although the evolutionary mechanisms differ, lineage-specific ORs are associated with ecological adaptations (**Figure 3**). The evolution of the mosquito *A. aegypti* OR4 illustrates how these evolutionary forces also act on a short time scale: alleles of OR4 in *A. aegypti* sub-species exhibit different sensitivities toward the cognate human odorant sulcatone, which is associated with human host preference (McBride et al., 2014). Mate selection is also driving odorant tuning in closely related noctuid species, where positive selection is acting on discrete amino-acid residues of PRs (Leary et al., 2012).

The constant tuning of ORs occurring over short and long time scales, as well as the conservation of specific lineages via purifying selection, runs counter to the notion that these receptors possess promiscuous binding sites. Such a broad peripheral filter would provide the central nervous system with the greater task of sorting out this crude input. However, there is little evidence that the CNS and the antennal lobe accomplish this task (Sachse and Galizia, 2003). In fact, one study has demonstrated that the discriminatory capabilities of the antennal lobes diminish when stimulated by high concentrations of odorants (Silbering et al., 2008). Rather, we concur with previous authors that ORs are better chemical detectors than previously assumed and that the burden of extracting behaviorally relevant odorant signals from the environment is largely their task (Hansson and Stensmyr, 2011). If so, the role of the antennal lobes would be to integrate olfactory inputs from hardwired lines associated with the detection of cognate odorants.

Molecular Receptive Range and Olfactory Tuning

The inherent advantages of the two-electrode voltage clamp system (Kvist et al., 2011) has facilitated the use of oocytes for assaying OR-odorant interactions (see References in Supplementary Table 1). Despite these efforts, the oocyte system is limited in the number of chemicals that can be tested and is most efficiently

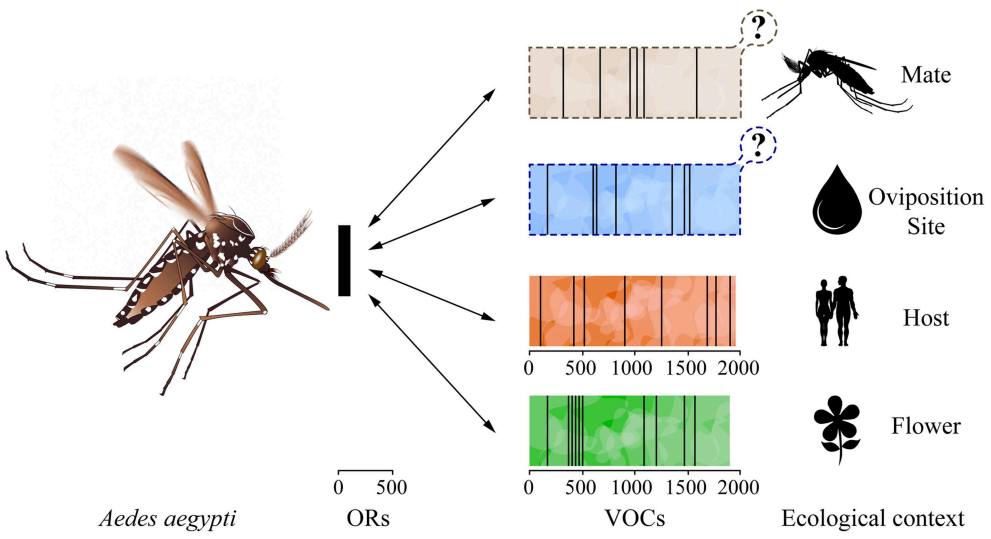

FIGURE 3 | Odorant receptors are molecular adaptations to various ecological niches. The mosquito *Aedes aegypti* may use up to 110 odorant receptors (ORs) to detect specific behaviorally relevant odorants (vertical black bars) emitted in the context of various ecological contexts. For illustration purposes, these cognate odorants are unknown and have been placed arbitrarily on the scale. The number of volatile organic compounds (VOCs) emitted by potential mates and oviposition sites remains largely unknown.

used for target validation studies or studies of receptor-ligand qualities such as concentration responsiveness and comparative efficacies (Kvist et al., 2011). Where oocytes have been used as a screen for previously uncharacterized insect ORs, dozens of receptors and tens of odorants have been used, providing hundreds of receptor–ligand comparisons (Wang et al., 2010). In stark contrast are functional studies in Human Embryonic Kidney cells, which can be used to screen tens of thousands of ligand–receptor combinations (Rinker et al., 2012). Both systems suffer from the inherent requirement to solubilize odorants in aqueous solutions, making it difficult to include VOCs that are often highly insoluble. This limitation can be circumvented by using an *in vivo* system such as the *Drosophila* empty neuron where VOCs can be delivered via airstream to OSNs expressing heterospecific ORs (Dobritsa et al., 2003), but again this system lacks truly high throughput capacity.

Two of the most critical limitations of OR heterologous functional screens relate to the lack of chemical ecological context. The first limitation is the unavoidable gap in *a priori* information regarding potentially meaningful relationships between ORs and their cognate ligands. This usually means that OR functional studies are carried out in more or less random fashion with ORs being targeted by VOCs that happen to be readily available.

The second limitation concerns the use of very high concentrations of odorants that may produce "hits;" i.e., VOCs that activate ORs with low probability of having any adaptive value (Dobritsa et al., 2003; Carey et al., 2010; Wang et al., 2010; Ray et al., 2014). This conundrum may not be of concern if the goal of the study is simply to identify chemicals that activate ORs and can be used at high concentrations for studies of channel properties or perhaps to identify drugs that alter insect behavior. Indeed the OR screens that have been carried out to date have been extremely valuable in elucidating the mechanisms of OR function and in providing a broader framework from which we can continue to refine our understanding of insect olfaction (reviewed in Suh et al., 2014). However, if a hypothesis-driven study depends on understanding ORs in the context of chemical ecology, random screening is unlikely to produce meaningful information. There are excellent examples of insect ORs that can be activated by numerous VOCs at low millimolar/high micromolar concentrations, which would tend to support the conclusion those receptors are broadly tuned (Hallem and Carlson, 2004, 2006; Kreher et al., 2008; Carey et al., 2010; Wang et al., 2010). However, some receptors in the same screens appear to be much more narrowly tuned when the VOC concentrations are reduced to low micromolar/high nanomolar range. This would be expected for a natural receptor-ligand pairing. For example, AgORs 2 and 10 exhibit broad molecular receptivity at high VOC concentrations but are in fact highly sensitive and narrowly tuned to their apparent cognate ligands, indole and skatole, respectively (Wang et al., 2010). Moreover, follow up studies with these receptors have validated the conclusions that these receptors display highly selective responses with low EC_{50}-values (Bohbot et al., 2011). These examples are analogous to a mammalian LGIC, the NMDA receptor, where glutamate is the endogenous ligand with high potency ($EC_{50} = 640$ nM), while a synthetic competitive agonist for which the receptor is named after exhibits a potency that is over an order of magnitude less potent ($EC_{50} = 12,000$ nM) (Nakanishi et al., 1992) (**Figure 2B**). In addition, the full activation of NMDA receptors *in vivo* requires the binding of glycine to an allosteric site and has a potency of 77 nM. These examples illustrate two mechanisms by which LGICs may display apparent broad molecular receptivity range: (i) high concentrations of non-meaningful orthosteric ligands and (ii) contributions of topographically distinct (allosteric) sites (**Figure 4A**). Interestingly, most studies of insect PRs in heterologous systems have not suffered from these kinds of biases, probably because their likely cognate ligands were previously known in pheromone blends (Nakagawa et al., 2005; Wanner et al., 2007, 2010; Mitsuno et al., 2008; Wang et al., 2011; Leary et al., 2012; Liu et al., 2013; Sun et al., 2013). One test of our theory on this topic would be to "screen" PRs with high concentrations of VOC libraries like the ones described in previous studies. We speculate that numerous chemicals would activate PRs in that situation, leading to the false perception that PRs are "generalists." Taken together, the limitations described here prescribe caution when interpreting the results of heterologous expression data and OR function. The dogma that many ORs are functional "generalists" has often been based on limited VOC libraries administered at high concentrations in heterologous systems. This problem is also encountered when screening ORNs *in vivo* using high doses (up to 10^{-2} dilutions) of odorants (Hallem et al., 2004; Hallem and Carlson, 2006; Carey et al., 2010). An important question to ask is whether one would expect to obtain such a high percentage of positive OR activating odorants among such small numbers of VOCs? In our opinion this seems unlikely and would lead us to conclude that such receptors in natural settings would be prone to activation by a potentially huge number of odorants that would render them quite useless at encoding meaningful information.

Conclusions

The physiological, pharmacological, evolutionary, and experimental arguments presented in this communication are part speculative and part empirical. Altogether, they support an idea that both ORs and NRs are specialized in the detection of evolutionary meaningful chemical cues and their distinction may remain based on their ecological context rather than on their pharmacological properties. To demonstrate this, we have provided evidence that (i) narrow tuning is not the exclusive attribute of PRs, (ii) GLVs and other non-pheromonal VOCs specifically activate nPRs, (iii) ORs are subjected to powerful selective pressures, and (iv) high concentrations of odorant stimuli cause broad non-specific OR responses.

Are ORs distributed on a continuum of tuning breadths made of a small number of narrowly tuned PRs and a majority of general nPRs (**Figure 4B**)? According to our analysis, ORs exhibit both broad molecular receptivity and narrow olfactory tuning. Insect ORs may be narrowly tuned to behaviorally relevant odorants but this selectivity can be overcome by overloading the system with high concentrations of chemicals carrying no adaptive value. Therefore, we distinguish the notions of molecular receptive range and olfactory tuning based on their chemical and ecological merits.

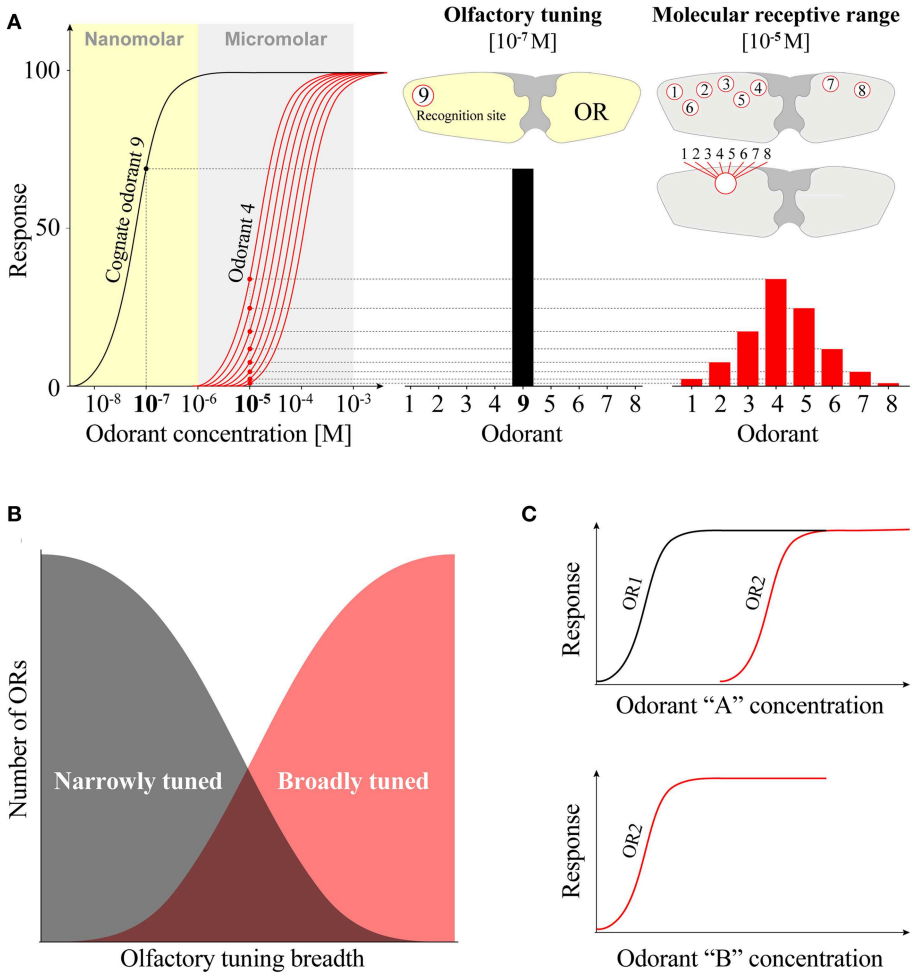

FIGURE 4 | Molecular receptive range and olfactory tuning of odorant receptors. (A) OR tuning curves represent OR responses to a set of odorants at a given concentration. Compared to odorant-response curves, tuning curves provide less resolution and sometime misleading information in terms of sensitivity and response profile. At high concentration (10^{-5} M), an odorant receptor (OR) is activated by odorants 1 through 8 thereby exhibiting a broad molecular receptive range (red histogram). These odorants may interact with an orthosteric site or in combination with multiple allosteric sites. At low concentration (10^{-7} M), the same OR is selectively activated by the cognate odorant 9 (black histogram) thus exhibiting a narrow olfactory tuning curve (black histogram). **(B)** Theoretical distributions of OR tuning breadths in insects. Deorphanization studies suggest that most ORs respond to many odorants while a smaller number are narrowly tuned (red curve). The identification of evolutionary meaningful OR-odorant pairings will shift this distribution toward a majority of ORs displaying narrow olfactory tuning breadth (black curve). **(C)** Odorant sensitivity of two paralog ORs, OR1, and OR2, respectively respond to the same odorant "A" with high (black curve) and low sensitivity (red curve), thereby endowing the peripheral olfactory system a greater response range. Alternatively, OR2 is narrowly tuned to cognate odorant "B."

While this hypothesis does not exclude the possibility that a few ORs exhibit broad olfactory tuning, it provides greater emphasis to the possibility that most ORs may be narrowly tuned (**Figure 4B**). Furthermore, we propose that nanomolar EC_{50}-values provide a criterion for insect OR deorphanization. If this is correct, the cognate ligands for *Bombyx mori* OR56 (Tanaka et al., 2009) and *Helicoverpa armigera* OR13 (Liu et al., 2013) remain to be identified (**Figure 2A**).

How do insects encode odorant intensity? It has been suggested that one of the mechanisms by which Drosophila ORs encode odorant intensity is by using paralogous OR pairs such as 42a and 42b, each detecting low and high amounts of the same odorant ligand (Kreher et al., 2008) (**Figure 4C**). This concept does not exclude the possibility that the response of an OR to high ligand concentrations rather indicates that the cognate ligand has yet to be identified. Such is the case with the mosquito indolergic receptors, OR2 and OR10, which respond to indole with high and low sensitivities, respectively (Bohbot et al., 2011). However, OR10 is narrowly tuned to skatole, a methylated analog of indole (Hughes et al., 2010; Bohbot and Dickens, 2012b). Perhaps more intriguing is the possibility that these two phenomena coexist. This is now a testable hypothesis using the aforementioned paralogous *Drosophila* ORs and looking for the cognate ligand of the low sensitivity receptor.

One of the greatest challenges in receptor neurobiology is the identification of OR-cognate odorant pairs. Based on our

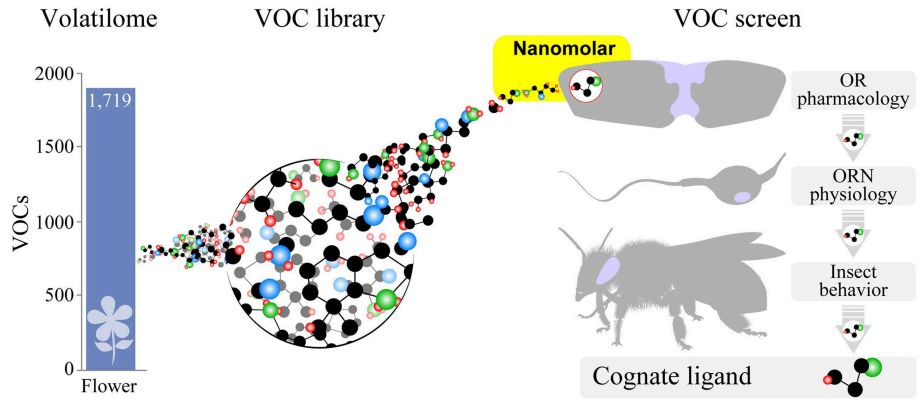

FIGURE 5 | Large-scale identification of behaviorally relevant volatile organic compounds. Animal and plant volatilomes may be used to generate a volatile organic compound (VOC) library for high throughput screening of odorant receptors (ORs) expressed in heterologous cell expression systems. VOCs exhibiting activity in the nanomolar range are further validated at the neurophysiological and behavioral levels.

analysis, we conclude that ORs and NRs are specialized receptors that selectively detect low concentrations of cognate ligands in the context of chemical communication. However, the data presented here is only a snapshot of the insect OR family. Among a few species, only a handful of ORs have been deorphanized, i.e., for which a cognate ligand has been identified. Matching host or plant volatilomes to an OR repertoire will not be trivial but would offer a comprehensive strategy to identify additional OR-cognate odorant pairs. Many of these VOCs have been published (Knudsen et al., 2006; de Lacy Costello et al., 2014) and may be used to generate a VOC library to test the response of heterologously expressed ORs (**Figure 5**) (Bohbot et al., 2014). A similar strategy, using gas chromatography-coupled single sensillum recording, has been applied to discover that ORNs are narrowly tuned to GLVs (Binyameen et al., 2014; Suer, 2014). Both approaches may provide lead bioactive compounds for behavioral validation.

The notions of generalist and specialist receptors were first proposed in 1964 by Schneider et al. (Schneider, 1964) in the context of ORN activation and were later applied to ORs as their underlying molecular mechanisms. Earlier authors had reported that GLV-detecting ORNs were as specialized as sex pheromone receptor neurons (Kafka, 1987; Dickens, 1990; Anderson and Hansson, 1995). Anderson recognized that the use of a limited number of GLVs for physiological screens prevented the identification of behaviorally relevant odorant ligands. Likewise, we have underlined that current OR deorphanization efforts use between 80 and 100 odorants, which is at least three-fold lower than the number of VOCs insects encounter in nature. The pharmacological data currently available reveal that PRs and nPRs exhibit comparable functional specialization provided the correct cognate ligands are known (Hansson and Stensmyr, 2011; Bengtsson et al., 2014).

The combination of short lifespan and narrow behavioral complexity in insects may explain the evolution of a peripheral olfactory system tuned to a narrow range of odorants involved with reproduction, mate selection and food selection, which do not exclude the possibility of associative learning. Interestingly, mammals can learn to detect chemicals devoid of adaptive value with high sensitivity and specificity. However, the comparatively oversized olfactory epithelium of mammals combined with their sniffing behavior may compensate for the inherent lack of sensitivity of its OR repertoire. Indeed, detector dogs seem to only detect the most abundant volatile chemicals in the headspace of explosives (Harper et al., 2005). On the other hand, mammals may have developed a peripheral olfactory system with higher tolerance for a greater variety of odorants amenable to odorant-based associations (Leon and Johnson, 2003; Wilson and Stevenson, 2003). It is more likely that the complexity of the central nervous system account for most of the plasticity required for associative learning (Mandairon and Linster, 2008).

There is no expedient method to test the theory proposed herein. Indeed, the identification of cognate odorants is time consuming and resource intensive. Since the pioneering studies on the deorphanization of insect ORs (Hallem et al., 2004; Carey et al., 2010; Wang et al., 2010), targeted functional studies have identified cognate ligands for nPRs and it is reasonable to expect that more will be identified. What remains to be understood is indeed staggering and will have important consequences in the fields of olfactory coding, medicine, and agriculture.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fevo.2015.00039/abstract

References

Amoore, J. E. (1963). Stereochemical theory of olfaction. *Nature* 198, 271–272. doi: 10.1038/198271a0

Anderson, P., and Hansson, B. S. (1995). Plant-odour-specific receptor neurones on the antennae of female and male *Spodoptera littoralis*. *Physiol. Entomol.* 20, 189–198. doi: 10.1111/j.1365-3032.1995.tb00001.x

Bengtsson, J. M., Gonzalez, F., Cattaneo, A. M., Montagné, N., Walker, W. B., Bengtsson, M., et al. (2014). A predicted sex pheromone receptor of codling moth *Cydia pomonella* detects the plant volatile pear ester. *Front. Ecol. Evol.* 2:33. doi: 10.3389/fevo.2014.00033

Binyameen, M., Anderson, P., Ignell, R., Birgersson, G., Razaq, M., Shad, S. A., et al. (2014). Identification of plant semiochemicals and characterization of new olfactory sensory neuron types in a polyphagous pest moth, *Spodoptera littoralis*. *Chem. Senses*. 39, 719–733. doi: 10.1093/chemse/bju046

Bohbot, J. D., and Dickens, J. C. (2009). Characterization of an enantioselective odorant receptor in the yellow fever mosquito *Aedes aegypti*. *PLoS ONE* 4:e7032. doi: 10.1371/journal.pone.0007032

Bohbot, J. D., and Dickens, J. C. (2012a). Selectivity of odorant receptors in insects. *Front Cell Neurosci.* 6:29. doi: 10.3389/fncel.2012.00029

Bohbot, J. D., and Dickens, J. C. (2012b). Odorant receptor modulation: ternary paradigm for mode of action of insect repellents. *Neuropharmacology* 62, 2086–2095. doi: 10.1016/j.neuropharm.2012.01.004.

Bohbot, J. D., Jones, P. L., Wang, G., Pitts, R. J., Pask, G. M., and Zwiebel, L. J. (2011). Conservation of indole responsive odorant receptors in mosquitoes reveals an ancient olfactory trait. *Chem. Senses* 36, 149–160. doi: 10.1093/chemse/bjq105

Bohbot, J. D., Strickman, D., and Zwiebel, L. J. (2014). The future of insect repellent discovery and development. *Outlook Pest Man.* 25, 265–270. doi: 10.1564/v25_aug_05

Bohbot, J., 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. doi: 10.1111/j.1365-2583.2007.00748.x

Burnstock, G. (2004). Cotransmission. *Curr. Opin. Pharmacol.* 4, 47–52. doi: 10.1016/j.coph.2003.08.001

Cande, J., Prud'homme, B., and Gompel, N. (2013). Smells like evolution: the role of chemoreceptor evolution in behavioral change. *Curr. Opin. Neurobiol.* 23, 152–158. doi: 10.1016/j.conb.2012.07.008

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. doi: 10.1038/nature08834

Chavez-Noriega, L. E., Crona, J. H., Washburn, M. S., Urrutia, A., Elliott, K. J., and Johnson, E. C. (1997). Pharmacological characterization of recombinant human neuronal nicotinic acetylcholine receptors h alpha 2 beta 2, h alpha 2 beta 4, h alpha 3 beta 2, h alpha 3 beta 4, h alpha 4 beta 2, h alpha 4 beta 4 and h alpha 7 expressed in *Xenopus* oocytes. *J. Pharmacol. Exp. Ther.* 280, 346–356.

Clements, J. D. (1996). Transmitter timecourse in the synaptic cleft: its role in central synaptic function. *Trends Neurosci.* 19, 163–171. doi: 10.1016/S0166-2236(96)10024-2

Croset, V., Rytz, R., Cummins, S. F., Budd, A., Brawand, D., Kaessmann, H., et al. (2010). Ancient protostome origin of chemosensory ionotropic glutamate receptors and the evolution of insect taste and olfaction. *PLoS Genet.* 6:e1001064. doi: 10.1371/journal.pgen.1001064

de Lacy Costello, B., Amann, A., Al-Kateb, H., Flynn, C., Filipiak, W., Khalid, T., et al. (2014). A review of the volatiles from the healthy human body. *J. Breath Res.* 8:014001. doi: 10.1088/1752-7155/8/1/014001

Dickens, J. C. (1990). Specialized receptor neurons for pheromones and host plant odors in the boll weevil, *Anthonomus grandis* Boh. (*Coleoptera: Curculionidae*). *Chem. Senses*. 15, 311–331. doi: 10.1093/chemse/15.3.311

Dobritsa, A., van der Goes van Naters, W., Warr, C., Steinbrecht, R., and Carlson, J. (2003). Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron* 37, 827–841. doi: 10.1016/S0896-6273(03)00094-1

Dudareva, N., Negre, F., Nagegowda, D. A., and Orlova, I. (2006). Plant volatiles: recent advances and future perspectives. *Crit. Rev. Plant Sci.* 25, 417–440. doi: 10.1080/07352680600899973

Faisal, A. A., Selen, L. P. J., and Wolpert, D. M. (2008). Noise in the nervous system. *Nat. Rev. Neurosci.* 9, 292–303. doi: 10.1038/nrn2258

Felten, S. Y., and Olschowka, J. (1987). Noradrenergic sympathetic innervation of the spleen: II. Tyrosine hydroxylase (TH)-positive nerve terminals form synapticlike contacts on lymphocytes in the splenic white pulp. *J. Neurosci. Res.* 18, 37–48. doi: 10.1002/jnr.490180108

Firestein, S. (2001). How the olfactory system makes sense of scents. *Nature* 413, 211–218. doi: 10.1038/35093026

Gardiner, A., Barker, D., and Butlin, R. (2008). *Drosophila* chemoreceptor gene evolution: selection, specialization and genome size. *Mol. Ecol.* 17, 1648–1657. doi: 10.1111/j.1365-294X.2008.03713.x

Große-Wilde, E., Gohl, T., Bouché, E., Breer, H., and Krieger, J. (2007). Candidate pheromone receptors provide the basis for the response of distinct antennal neurons to pheromonal compounds. *Eur. J. Neurosci.* 25, 2364–2373. doi: 10.1111/j.1460-9568.2007.05512.x

Hallem, E. A., and Carlson, J. R. (2004). The odor coding system of *Drosophila*. *Trends Genet.* 20, 453–459. doi: 10.1016/j.tig.2004.06.015

Hallem, E. A., and Carlson, J. R. (2006). Coding of odors by a receptor repertoire. *Cell* 125, 143–160. doi: 10.1016/j.cell.2006.01.050

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. doi: 10.1016/j.cell.2004.05.012

Hansson, B. S., and Stensmyr, M. C. (2011). Evolution of insect olfaction. *Neuron* 72, 698–711. doi: 10.1016/j.neuron.2011.11.003

Harper, R. J., Almirall, J. R., and Furton, K. G. (2005). Identification of dominant odor chemicals emanating from explosives for use in developing optimal training aid combinations and mimics for canine detection. *Talanta* 67, 313–327. doi: 10.1016/j.talanta.2005.05.019

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. doi: 10.1126/science.1076196

Hughes, D. T., Pelletier, J., Luetje, C. W., and Leal, W. S. (2010). Odorant receptor from the southern house mosquito narrowly tuned to the oviposition attractant skatole. *J. Chem. Ecol.* 36, 797–800. doi: 10.1007/s10886-010-9828-9

Kafka, W. A. (1987). Similarity of reaction spectra and odor discrimination – Single receptor cell recordings in *Antheraea polyphemus* (Saturniidae). *J. Comp. Physiol. A* 161, 867–880. doi: 10.1007/BF00610228

Karayannis, T., Elfant, D., Huerta-Ocampo, I., Teki, S., Scott, R. S., Rusakov, D. A., et al. (2010). Slow GABA transient and receptor desensitization shape synaptic responses evoked by hippocampal neurogliaform cells. *J. Neurosci.* 30, 9898–9909. doi: 10.1523/JNEUROSCI.5883-09.2010

Knudsen, J. T., Eriksson, R., Gershenzon, J., and Ståhl, B. (2006). Diversity and distribution of floral scent. *Bot. Rev.* 72, 1–120. doi: 10.1663/0006-8101(2006)72[1:DADOFS]2.0.CO;2

Kreher, S. A., Mathew, D., Kim, J., and Carlson, J. R. (2008). Translation of sensory input into behavioral output via an olfactory system. *Neuron* 59, 110–124. doi: 10.1016/j.neuron.2008.06.010

Kuffler, S. W., and Yoshikami, D. (1975). The number of transmitter molecules in a quantum: an estimate from iontophoretic application of acetylcholine at the neuromuscular synapse. *J. Physiol.* 251, 465–482. doi: 10.1113/jphysiol.1975.sp011103

Kvist, T., Hansen, K. B., and Bräuner-Osborne, H. (2011). The use of *Xenopus* oocytes in drug screening. *Expert Opin. Drug Discov.* 6, 141–153. doi: 10.1517/17460441.2011.546396

Leary, G. P., Allen, J. E., Bunger, P. L., Luginbill, J. B., Linn, C. E., Macallister, I. E., et al. (2012). Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14081–14086. doi: 10.1073/pnas.1204661109

Leon, M., and Johnson, B. A. (2003). Olfactory coding in the mammalian olfactory bulb. *Brain Res. Rev.* 42, 23–32. doi: 10.1016/S0165-0173(03)00142-5

Liu, C., Liu, Y., Guo, M., Cao, D., Dong, S., and Wang, G. (2014). Narrow tuning of an odorant receptor to plant volatiles in *Spodoptera exigua* (Hübner). *Insect Mol. Biol.* 23, 487–496. doi: 10.1111/imb.12096

Liu, Y., Liu, C., Lin, K., and Wang, G. (2013). Functional specificity of sex pheromone receptors in the cotton bollworm *Helicoverpa armigera*. *PLoS ONE* 8:e62094. doi: 10.1371/journal.pone.0062094

Malnic, B., Hirono, J., Sato, T., and Buck, L. B. (1999). Combinatorial receptor codes for odors. *Cell* 96, 713–723. doi: 10.1016/S0092-8674(00)80581-4

Mandairon, N., and Linster, C. (2008). Odor perception and olfactory bulb plasticity in adult Mammals. *J. Neurophysiol.* 101, 2204–2209. doi: 10.1152/jn.00076.2009

McBride, C. (2007). Rapid evolution of smell and taste receptor genes during host specialization in *Drosophila sechellia. Proc. Natl. Acad. Sci. U.S.A.* 104, 4996–5001. doi: 10.1073/pnas.0608424104

McBride, C. S., Arguello, J. R., and O'Meara, B. C. (2007). Five *Drosophila* genomes reveal nonneutral evolution and the signature of host specialization in the chemoreceptor superfamily. *Genetics* 177, 1395–1416. doi: 10.1534/genetics.107.078683

McBride, C. S., Baier, F., Omondi, A. B., Spitzer, S. A., Lutomiah, J., Sang, R., et al. (2014). Evolution of mosquito preference for humans linked to an odorant receptor. *Nature* 515, 222–227. doi: 10.1038/nature13964

Missbach, C., Dweck, H. K., Vogel, H., Vilcinskas, A., Stensmyr, M. C., Hansson, B. S., et al. (2014). Evolution of insect olfactory receptors. *Elife* 3:e02115. doi: 10.7554/eLife.02115

Mitsuno, H., Sakurai, T., Murai, M., Yasuda, T., Kugimiya, S., Ozawa, R., et al. (2008). Identification of receptors of main sex-pheromone components of three *Lepidopteran* species. *Eur. J. Neurosci.* 28, 893–902. doi: 10.1111/j.1460-9568.2008.06429.x

Miura, N., Nakagawa, T., Tatsuki, S., Touhara, K., and Ishikawa, Y. (2009). A male-specific odorant receptor conserved through the evolution of sex pheromones in *Ostrinia moth* species. *Int. J. Biol. Sci.* 5, 319–330. doi: 10.7150/ijbs.5.319

Moncrieff, R. W. (1949). What is odor? A new theory. *Am. Perfumer*. 54, 453–454.

Nakagawa, T., Sakurai, T., Nishioka, T., and Touhara, K. (2005). Insect sex-pheromone signals mediated by specific combinations of olfactory receptors. *Science* 307, 1638–1642. doi: 10.1126/science.1106267

Nakanishi, N., Axel, R., and Shneider, N. A. (1992). Alternative splicing generates functionally distinct N-methyl-D-aspartate receptors. *Proc. Natl. Acad. Sci. U.S.A.* 89, 8552–8556. doi: 10.1073/pnas.89.18.8552

Neafsey, D. E., Waterhouse, R. M., Abai, M. R., Aganezov, S. S., Alekseyev, M. A., Allen, J. E., et al. (2015). Highly evolvable malaria vectors: the genomes of 16 *Anopheles* mosquitoes. *Science* 347, 1258522–1258522. doi: 10.1126/science.1258522

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. doi: 10.1038/nrg2480

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. doi: 10.1371/journal.pone.0010090

Penn, D. J., Oberzaucher, E., Grammer, K., Fischer, G., Soini, H. A., Wiesler, D., et al. (2007). Individual and gender fingerprints in human body odour. *J. R. Soc. Interface* 4, 331–340. doi: 10.1098/rsif.2006.0182

Phillips, M. (1997). Method for the collection and assay of volatile organic compounds in breath. *Anal. Biochem.* 247, 272–278. doi: 10.1006/abio.1997.2069

Phillips, M., Cataneo, R. N., Chaturvedi, A., Kaplan, P. D., Libardoni, M., Mundada, M., et al. (2013). Detection of an extended human volatome with comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry. *PLoS ONE* 8:e75274. doi: 10.1371/journal.pone.0075274

Ray, A., van Naters, W. G., and Carlson, J. R. (2014). Molecular determinants of odorant receptor function in insects. *J. Biosci.* 39, 555–563. doi: 10.1007/s12038-014-9447-7

Rinaldi, A. (2007). The scent of life. *EMBO Rep.* 8, 629–633. doi: 10.1038/sj.embor.7401029

Rinker, D. C., Jones, P. L., Pitts, R. J., Rutzler, M., Camp, G., Sun, L., et al. (2012). Novel high-throughput screens of *Anopheles gambiae* odorant receptors reveal candidate behaviour-modifying chemicals for mosquitoes. *Physiol. Entomol.* 37, 33–41. doi: 10.1111/j.1365-3032.2011.00821.x

Robertson, H. M., and Wanner, K. W. (2006). The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. *Genome Res.* 16, 1395–1403. doi: 10.1101/gr.5057506

Sachse, S., and Galizia, C. G. (2003). The coding of odour-intensity in the honeybee antennal lobe: local computation optimizes odour representation. *Eur. J. Neurosci.* 18, 2119–2132. doi: 10.1046/j.1460-9568.2003.02931.x

Sánchez-Gracia, A., Vieira, F. G., and Rozas, J. (2009). Molecular evolution of the major chemosensory gene families in insects. *Heredity* 103, 208–216. doi: 10.1038/hdy.2009.55

Sato, K., Tanaka, K., and Touhara, K. (2011). Sugar-regulated cation channel formed by an insect gustatory receptor. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11680–11685. doi: 10.1073/pnas.1019622108

Schneider, D. (1964). Insect antennae. *Ann. Rev. Entomol.* 9, 103–122. doi: 10.1146/annurev.en.09.010164.000535

Silbering, A. F., Okada, R., Ito, K., and Galizia, C. G. (2008). Olfactory information processing in the *Drosophila* antennal lobe: anything goes? *J. Neurosci.* 28, 13075–13087. doi: 10.1523/JNEUROSCI.2973-08.2008

Smit, A. B., Syed, N. I., Schaap, D., van Minnen, J., Klumperman, J., Kits, K. S., et al. (2001). A glia-derived acetylcholine-binding protein that modulates synaptic transmission. *Nature* 411, 261–268. doi: 10.1038/35077000

Stensmyr, M. C., Dweck, H., Farhan, A., Ibba, I., and Strutz, A. (2012). A Conserved dedicated olfactory circuit for detecting harmful microbes in *Drosophila.* Cell 151, 1345–1357. doi: 10.1016/j.cell.2012.09.046

Stocker, R., and Nuesch, H. (1975). Ultrastructural studies on neuromuscular contacts and the formation of junctions in the flight muscle of *Antheraea polyphemus* (Lep.) I. Normal adult development. *Cell Tissue Res.* 159, 245–266. doi: 10.1007/BF00219160

Suer, R. A. (2014). *Unravelling the Malaria Mosquito's Sense of Smell: Neural and Behavioural Responses to Human-Derived Compounds*, 2011, Ph.D. Thesis, Wageningen University.

Suh, E., Bohbot, J., and Zwiebel, L. J. (2014). Peripheral olfactory signaling in insects. *Curr. Opin. Insect Sci.* 6, 86–92. doi: 10.1016/j.cois.2014.10.006

Sun, M., Liu, Y., Walker, W. B., Liu, C., Lin, K., Gu, S., et al. (2013). Identification and characterization of pheromone receptors and interplay between receptors and pheromone binding proteins in the diamondback moth, *Plutella xyllostella*. *PLoS ONE* 8:e62098. doi: 10.1371/journal.pone.0062098

Tanaka, K., Uda, Y., Ono, Y., Nakagawa, T., Suwa, M., Yamaoka, R., et al. (2009). Highly selective tuning of a silkworm olfactory receptor to a key mulberry leaf volatile. *Curr. Biol.* 19, 881–890. doi: 10.1016/j.cub.2009.04.035

Vogt, R., and Riddiford, L. (1981). Pheremone binding and inactivation by moth antennae. *Nature* 293, 161–163. doi: 10.1038/293161a0

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. U.S.A.* 107, 4418–4423. doi: 10.1073/pnas.0913392107

Wang, G., Vásquez, G. M., Schal, C., Zwiebel, L. J., and Gould, F. (2011). Functional characterization of pheromone receptors in the tobacco budworm *Heliothis virescens. Insect Mol. Biol.* 20, 125–133. doi: 10.1111/j.1365-2583.2010.01045.x

Wanner, K. W., Nichols, A. S., Allen, J. E., Bunger, P. L., Garczynski, S. F., Linn, C. E., et al. (2010). Sex pheromone receptor specificity in the European corn borer moth, *Ostrinia nubilalis*. *PLoS ONE* 5:e8685. doi: 10.1371/journal.pone.0008685

Wanner, K. W., Nichols, A. S., Walden, K. K. O., Brockmann, A., Luetje, C. W., and Robertson, H. M. (2007). A honey bee odorant receptor for the queen substance 9-oxo-2-decenoic acid. *Proc. Natl. Acad. Sci. U.S.A.* 104, 14383–14388. doi: 10.1073/pnas.0705459104

Wilson, D. A., and Stevenson, R. J. (2003). Olfactory perceptual learning: the critical role of memory in odor discrimination. *Neurosci. Biobehav. Rev.* 27, 307–328. doi: 10.1016/S0149-7634(03)00050-2

Wonnacott, S., and Barik, J. (2007). Nicotinic ACh receptors. *Tocris Biosci. Sci. Rev.* 28, 1–20.

Xu, P., Garczynski, S. F., Atungulu, E., Syed, Z., Choo, Y.-M., Vidal, D. M., et al. (2012). Moth sex pheromone receptors and deceitful parapheromones. *PLoS ONE* 7:e41653. doi: 10.1371/journal.pone.0041653

Zhou, X., Slone, J. D., Rokas, A., Berger, S. L., Liebig, J., Ray, A., et al. (2012). Phylogenetic and transcriptomic analysis of chemosensory receptors in a pair of divergent ant species reveals sex-specific signatures of odor coding. *PLoS Genet.* 8:e1002930. doi: 10.1371/journal.pgen.1002930

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Insect olfaction and the evolution of receptor tuning

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Insects detect odorants primarily using odorant receptors (OR) housed in the dendritic membrane of olfactory sensory neurons (OSN). Pioneering studies indicated that insects, like mammals, detect odorants in a combinatorial fashion with a specific odor ligand activating several broadly tuned ORs, and each OR being activated by several ligands. Several recent studies, however, challenge this view by providing examples where ecologically relevant odorants are detected by high-specificity ORs activating dedicated neuronal circuits. Here we review these contrasting findings on the ligand selectivity of insect ORs and their neuronal wiring, and outline scenarios describing how adaptive and neutral evolution might shape both narrow and broad receptor tuning. The fact that not all ORs display narrow tuning might partly be due to key ligands having been missed from screens or too high stimuli concentrations being used. However, the birth-and-death model of OR evolution, involving both adaptive and neutral events, could also explain the evolution of broad tuning in certain receptors due to positive selection or relaxed constraint. If the insect olfactory system indeed contains both narrowly and broadly tuned ORs, this suggests that it is a hybrid between dedicated channels and combinatorial coding. The relative extent of the two coding modes is then likely to differ between species, depending on requirements of perceived chemical space and the size of the OR repertoire. We address this by outlining scenarios where certain insect groups may be more likely to have evolved combinatorial coding as their dominant coding strategy. Combinatorial coding may have evolved predominantly in insects that benefit from the ability to discriminate between a larger number of odorants and odor objects, such as polyphagous or social species. Alternatively, combinatorial coding may have evolved simply as a mechanism to increase perceived odor space in species with small OR repertoires.

Keywords: combinatorial coding, evolution, olfaction, odorant receptor, selectivity, specificity

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Introduction

Animals across phyla detect odors from the surrounding environment to find food, hosts, mates and oviposition sites, and to avoid predators and pathogens (Hildebrand and Shepherd, 1997). Thus, the sense of smell is often critically important for survival and arguably linked to fitness, having evolved to fit various ecological lifestyles (Nei et al., 2008; Hansson and Stensmyr, 2011; Cande et al., 2013). Animals detect odorants using several families of receptor proteins, of which the odorant receptors (ORs) are the most thoroughly studied in terms of function. Early studies

on mammals suggested that their ORs respond to odorants in a combinatorial fashion, with a specific odor ligand activating multiple ORs, and each OR being activated by multiple ligands (**Figure 1A**). This in turn results in a combinatorial activation of olfactory sensory neurons (OSN) in the periphery and glomeruli in the primary olfactory center (**Figure 1B**) (Malnic et al., 1999; but see Wetzel et al., 1999; Keller et al., 2007; Nara et al., 2011; Shirasu et al., 2014). By using promiscuous receptors with overlapping response spectra, an animal is expected to be able to discriminate among more odorants and odor blends through central processing of the signals than predicted by the number of receptors it expresses (e.g., Xu et al., 2000; Bushdid et al., 2014).

Pioneering functional studies on the OR repertoire of *Drosophila melanogaster* (Hallem et al., 2004; Kreher et al., 2005; Hallem and Carlson, 2006), and later of mosquitoes (Xia et al., 2008; Carey et al., 2010; Wang et al., 2010), suggested that insect ORs respond to multiple ligands (i.e., are broadly tuned), and that combinatorial coding also likely applies to insect olfaction, with their modest repertoires of receptors and glomeruli. Based on these findings, combinatorial coding has been established as the predominant explanation of how insects "encode" their odor environments (e.g., Touhara and Vosshall, 2009; Wyatt, 2014). However, several recent studies challenge this view by providing examples where certain odors are detected by highly specific receptors (Mathew et al., 2013; Liu et al., 2014) (**Figure 1A**), activating dedicated neuronal pathways (originally referred to as "labeled-line" channels) (**Figure 1B**) (Stensmyr et al., 2012; Dweck et al., 2013, 2015; Ronderos et al., 2014). In light of these findings, we review the literature on ligand selectivity of insect ORs, and outline ecological and evolutionary scenarios that, in combination with neurological constraints, we believe are likely to have favored high specificity of the insect ORs and their corresponding sensory neurons. We also discuss how adaptive and neutral evolution might allow some receptors to acquire broad tuning, facilitating combinatorial coding in certain

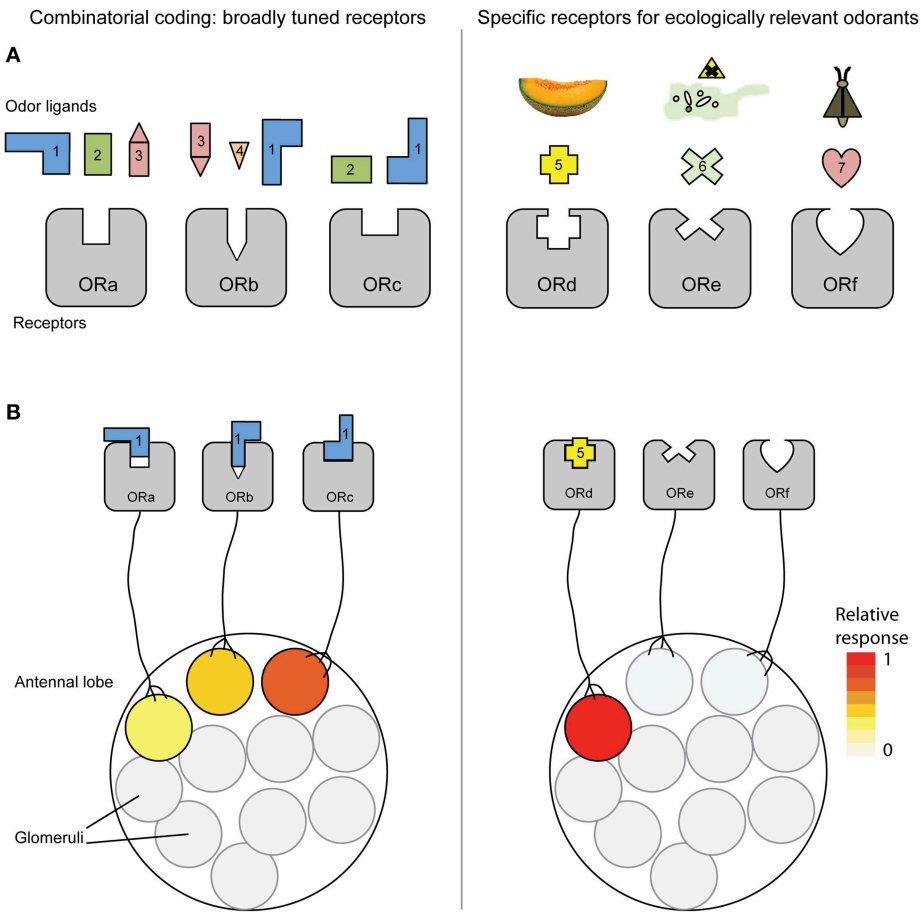

FIGURE 1 | Combinatorial coding including broadly tuned odorant receptors (OR) contrasted with specialized ORs activating dedicated neuronal pathways. (A) With combinatorial coding (left), each odorant binds to several receptors with overlapping ligand affinities, and each OR is activated by multiple ligands. In contrast, specific ORs for ecologically relevant cues are activated by one or a few structurally related odorants, and little cross-activation of ORs occurs (right). **(B)** With combinatorial coding, a single odorant activates a unique combination of glomeruli in the antennal lobe (left) due to binding to multiple ORs, whereas a single odorant activates a single glomerulus in a system comprised of specialized ORs and dedicated channels (right).

olfactory sub-systems. Finally, we present our views on in which insect groups one would expect combinatorial coding to be more prevalent.

Definition of Combinatorial Coding

Before venturing into the discussion about the different odor coding modes, it is important to clearly state our working definition of "combinatorial coding," because its meaning is not consistent in the literature. Originally, the definition of combinatorial coding was as described above: each OR is broadly tuned, responding to multiple ligands, and single ligands activate multiple ORs and as a consequence also multiple glomeruli in the primary olfactory center (Malnic et al., 1999). This is the definition we employ here. However, combinatorial coding is also used to describe central processing mechanisms, and can in this case, actually involve receptors specific for single compounds (Wyatt, 2014). For instance, moth pheromone receptors often respond specifically to individual pheromone components, while blend-specific antennal lobe (AL) interneurons and projection neurons might need combinatorial input from several of these OSNs to respond (Christensen et al., 1989; Wu et al., 1996). In general, insects rely on specific combinations and ratios of odorants for behavioral responses, such as host attraction and non-host avoidance (Bruce et al., 2005; Bruce and Pickett, 2011; Binyameen et al., 2014; Riffell et al., 2014; Thoma et al., 2014). Thus, at the level of odor objects, such as host plants releasing a bouquet of odorants, combinatorial coding is operating both at the periphery and in the AL (Semmelhack and Wang, 2009), because the individual constituents of the blend activate different neurons, regardless of whether the ORs they express are specific or promiscuous. This is, however, distinct from combinatorial coding with respect to single odor ligands, which is the focus of the present review.

Experimental Factors Affecting Tuning Widths and Combinatorial Responses

Selection of Test Compounds and Stimuli Concentrations

There are several experimental factors that should be considered when interpreting the tuning width of receptors. For instance, the number and selection of test odorants can influence apparent response specificities, thus also the kurtosis (k) value of ORs, which sometimes is used as a numerical representation of tuning width based on the "peakedness" of the odor response distribution (e.g., Carey et al., 2010). The response profile of a receptor will, in general, appear broader (lower k) if the odor panel contains a larger number of compounds that are chemically related to the primary ligand, as compared to if the test panel contains a smaller number or is devoid of such compounds. Thus, a biased coverage of chemical space in test odor panels will inevitably bias apparent response specificities in a receptor-dependent manner. In our schematic examples of tuning widths (i.e., see **Figures 2**, **3**) we do not consider chemical space coverage when illustrating the difference between narrowly and broadly tuned receptors.

The applied stimulus concentration is a second factor that greatly influences response specificities. The first large-scale heterologous screenings of the OR repertoires of *D. melanogaster* and *A. gambiae* revealed a wide range of OR specificities, from narrow to broad tuning (Kreher et al., 2005; Hallem and Carlson, 2006; Xia et al., 2008; Carey et al., 2010; Wang et al., 2010). Since most ORs were activated by multiple ligands and most ligands activated multiple ORs, it was concluded that combinatorial coding is the primary coding mode of the insect olfactory system. This conclusion is well supported by the data at that time. For example, of the 24 *D. melanogaster* ORs studied by Hallem and Carlson (2006), 16 responded strongly (>100 spikes/s) to multiple odorants within a panel of 110 test odorants with variable chemistry. Seven of the other ORs were only weakly (<100 spikes/s) excited, whereas one OR responded strongly only to one odorant. In this study, 67 of the odorants excited more than one OR. However, the stimulus concentrations used (10^{-4}–10^{-2} dilution) in the early studies on the fly and the mosquito were probably higher than those insects generally would encounter in nature, although the local concentrations close to the sensilla have not been determined. Importantly, it is the natural odor concentrations that exert the selection pressures that shape the OR specificities. Indeed, at lower stimulus loads, the ORs are more narrowly tuned (Hallem and Carlson, 2006; Kreher et al., 2008; Wang et al., 2010), a phenomenon that has also been observed in single-unit electrophysiological recordings from a large variety of insect species (e.g., Hansson et al., 1999; Andersson et al., 2009, 2012a; Yuvaraj et al., 2013). Hence, the broad response of receptors leading to the conclusion of combinatorial activation of insect ORs might in some cases be a pharmacological effect on ORs when exposed to high concentrations of chemicals, thus not always reflecting how the repertoire of receptors is used in nature or how it evolved. This observation has now been recognized by the research group that conducted the pioneering studies on *D. melanogaster* (Mathew et al., 2013) (see also Section Specific Receptors and Dedicated Neuronal Channels in *D. melanogaster*).

Measurements of Stimuli Quantities and Purities are Essential

There are additional technical issues that may confound conclusions about receptor tuning. In order to conclude that a receptor or OSN is broadly tuned, it is important to measure the actual amount of stimulus that reaches the sensory cell (or at least the sensory organ), especially if the active compounds are structurally unrelated and have different volatilities (airborne odor delivery systems) or solubilities (liquid-borne systems). These issues are usually not thoroughly considered when analyzing the data from physiological studies. Andersson et al. (2012b) showed that differences between stimuli in their release rates from commonly used Pasteur pipette odor cartridges can significantly confound conclusions about OR specificities, and used the *D. melanogaster* ab3A neuron, co-expressing *Or22a* and *Or22b* (Dobritsa et al., 2003), as an example. The ab3A neuron had previously been shown to respond to several odorants and with similar sensitivity to the three proposed key ligands, methyl hexanoate, ethyl hexanoate, and ethyl butyrate (Stensmyr

et al., 2003; Hallem and Carlson, 2006; Pelz et al., 2006). However, these compounds have vastly different release rates from odor cartridges (Andersson et al., 2012b). When correcting for airborne stimuli quantities, the ab3A neuron has the highest sensitivity to ethyl hexanoate, with a response threshold at a dose one order of magnitude lower than for methyl hexanoate and four orders of magnitude lower than for ethyl butyrate, indicating a higher specificity than previously acknowledged (Andersson et al., 2012b). Furthermore, Andersson et al. (2012b) also highlights the importance of the purity of the synthetic test compounds, which is rarely properly analyzed and reported (but see e.g., Andersson et al., 2009). Without careful consideration it is possible to arrive at false conclusions due to the presence of active contaminants. Even compounds of the highest quality are seldom more than 99% pure, and the slightest impurity of an active and highly volatile compound (e.g., 0.001% of ethyl butyrate in the ethyl hexanoate vial) could elicit a significant "false positive" response from a sensitive receptor.

Specific Receptors for Compounds Linked to Fitness

It has long been known that insect OSNs that respond to sex or aggregation pheromone compounds often are highly specific (Mustaparta et al., 1979; Tømmerås, 1985; Hansson et al., 1986; Priesner, 1986; Almaas et al., 1991). More recent screenings of receptors in heterologous systems have confirmed that the specificity generally resides in the pheromone receptors housed in these neurons (Wanner et al., 2007, 2010; Mitchell et al., 2012; Montagné et al., 2012; Zhang and Löfstedt, 2013; Jiang et al., 2014). High specificity of pheromone detection is likely to be maintained due to strong purifying selection on the receptors to keep high fidelity in the mate recognition system (**Figure 2A**), ensuring reproductive success (e.g., Leary et al., 2012). In other words, mutations in pheromone receptors that widen or alter the ligand specificity would generally be deleterious, potentially leading to reduced mating efficiency or heterospecific mating, thus lower fitness. Moreover, in recent years it has become apparent that OSNs responding to non-pheromonal compounds can also be highly selective, including those responding to ubiquitous plant volatiles (reviewed in e.g., Bruce and Pickett, 2011; Hansson and Stensmyr, 2011). Evidence from a variety of insect species suggests that a high degree of selectivity is common also for receptors that detect non-pheromonal compounds important for survival and reproduction. Discussed below is data indicating a high degree of ligand specificity for over a third of the ORs encoded within the genome of *D. melanogaster*.

Specific Receptors and Dedicated Neuronal Channels in *D. melanogaster*

There are several examples of specifically tuned ORs that detect ecologically relevant odorants to activate dedicated neuronal channels in *D. melanogaster*. Stensmyr et al. (2012) identified a dedicated olfactory circuit in flies for geosmin. This compound signals the presence of toxic molds and bacteria on their breeding substrates, fermenting fruit. By using GC-coupled electrophysiology, the authors tested ca. 3000 compounds that are present in the fly's natural environment, and showed that only the ab4B neuron, which expresses *Or56a*, is activated exclusively by geosmin. Subsequent recordings from Chinese Hamster Ovary cells showed that OR56a is extremely specific for geosmin. Furthermore, activation of the ab4B neuron by geosmin elicits activity in a single glomerulus (DA2), which is necessary and sufficient for antifeeding activity, inhibition of attraction and oviposition. Geosmin-specific OSNs were identified in seven other fruit-breeding drosophilids, indicating the evolutionary conservation of geosmin detection. It was also shown that the receptor is subjected to purifying selection. However, *D. elegans* did not respond to geosmin, and the fact that this species breeds on fresh flowers, and therefore is unlikely to encounter the toxic molds, suggests that the link between geosmin and reduced fitness is much weaker in this species (Stensmyr et al., 2012).

Another dedicated olfactory pathway of *D. melanogaster* is activated by the binding of citrus volatiles (primarily valencene) to the receptor OR19a (Dweck et al., 2013). It was shown that flies prefer citrus fruit as an oviposition substrate, and that the terpenes characteristic of these fruits are detected solely by OSNs expressing *Or19a*. Activation of these neurons was necessary and sufficient for the oviposition selectivity, but did not induce long-range attraction. Moreover, endoparasitoid wasps that parasitize fly larvae were repelled by citrus odors, and fly larvae had a reduced risk of parasitism in the presence of valencene. Thus, although the OR19a receptor is more broadly tuned than the receptor for geosmin, it still activates a dedicated neuronal circuit because it responds only to terpene compounds associated with the preferred oviposition substrate, and these compounds do not excite other OSN classes. Another recent study showed that DmelOR83c is specific for farnesol, which is released from the peal of citrus fruit (Ronderos et al., 2014). In contrast to the OR19a pathway, activation of farnesol-sensitive OSNs induces attraction. This might suggest two distinct pathways of which one (via OR83c) is essential for citrus attraction and the other (via OR19a) for citrus oviposition preference (Dweck et al., 2013; Ronderos et al., 2014). However, the ecological significance of farnesol and the OR83c pathway was recently questioned by Mansourian and Stensmyr (2015). Farnesol is present in minor quantities only in some citrus varieties, and the fact that the projection neurons from the glomerulus that receives input from *Or83c* expressing OSNs terminate in the brain area that processes pheromonal information might imply that this receptor simply is an orphan pheromone receptor (as discussed in Mansourian and Stensmyr, 2015).

D. melanogaster also responds to hydroxycinnamic acids (HCAs), potent dietary antioxidants common in fruits, using the detection of ethylphenols (primarily 4-ethylguaiacol and 4-ethylphenol) as a proxy (Dweck et al., 2015). Being able to detect foods containing dietary antioxidants is likely to be beneficial for flies and other generalist insects to counteract acute oxidative stress induced by consumption of entomopathogenic microorganisms (as argued by Dweck et al., 2015). The ethylphenols are produced from HCAs by several yeast species commonly found on fruit, and specifically detected by adult flies solely by the OSN class expressing *Or71a*. This OSN class was screened with 154 compounds at a high dose (10^{-2} dilution)

to identify its maximum receptive range. In this screen, 4-ethylguaiacol elicited the strongest responses, and only eight other compounds, all structurally similar to the primary ligand, elicited responses at >100 spikes/s. Furthermore, activation of this neuron class was necessary and sufficient for increased feeding, oviposition and attraction. Also, in fly larvae, a similar dedicated neuronal pathway mediates positive chemotaxis to HCA-enriched substrates via proxy detection of the same ethylphenols. Interestingly, this pathway is activated by OSNs expressing the larval receptor OR94b, which has a response spectra very similar to that of OR71a in adults (Dweck et al., 2015).

In addition to the examples above, Mathew et al. (2013) screened the 21 larval-expressed ORs of *D. melanogaster* with close to 500 odorants at a dilution of 10^{-4}. The test odor panel was comprised of chemically diverse compounds, including aldehydes, ketones, esters, aromatics, alcohols, terpenes, and pyrazines. Many of the compounds are released from natural sources such as yeast, fruits, and fungi. Odor-evoked responses were obtained from 19 of the ORs, and the active compounds elicited little cross-activation of other ORs. It was concluded that naturally occurring concentrations of many of the test odorants are likely to be signaled by single OSN classes, thus not by combinatorial activation of several neurons. Furthermore, the majority of the key ligands for the 19 ORs had effects on larval behavior (various degrees of attraction). However, the ecological relevance of each specific compound was not determined and the high response thresholds for some of the ORs to their key ligands (between 10^{-2} and 10^{-3} dilutions) indicate that they are more sensitive to other unidentified ligands (Mathew et al., 2013).

Receptor Specificity in Other Species and Receptor Families

High specificity of ORs for non-pheromonal compounds has also been found in mosquitoes, such as ORs detecting host and oviposition attractants. Lu et al. (2007) screened 97 compounds to identify *A. gambiae* OR8 as a specific detector for 1-octen-3-ol, a compound characteristic of humans and large mammalian herbivores. In *Aedes aegypti*, the OR8 ortholog was subsequently shown to primarily respond to the (*R*)-(−)-enantiomer of the compound (Bohbot and Dickens, 2009). In *Culex quinquefasciatus*, the oviposition attractants indole (Pelletier et al., 2010) and skatole (Hughes et al., 2010) are detected by specific receptors (OR2 and OR10, respectively), although only 23 compounds were tested in these two studies (see also Bohbot and Dickens, 2012; Bohbot and Dickens, and references therein). In the beet armyworm moth (*Spodoptera exigua*), SexiOR3 was found to be specific for (*E*)-(β)-farnesene within a panel of 62 odorants tested (Liu et al., 2014). Only four other compounds with very similar chemical structure elicited minor responses from this receptor. Similarly in the codling moth (*Cydia pomonella*), the plant compound pear ester ((*E*,*Z*)-2,4-decadienoate), which is a powerful pheromone synergist, was detected specifically by CpomOR3 (Bengtsson et al., 2014). However, the test odor panel only included 15 compounds, mostly pheromone compounds and antagonists from related species. Thus, this OR might show a broader response if tested with an expanded and more diverse set of odor stimuli.

In addition to OSNs that harbor ORs, dedicated olfactory pathways, which are fed by input from OSNs expressing ionotropic receptors (IR) or gustatory receptors (GR), have been identified in *Drosophila*. Examples include avoidance of carbon dioxide via GR21a and GR63a (co-expressed in the same OSN) (Jones et al., 2007; Kwon et al., 2007), aversion toward specific acids via IR64a (Ai et al., 2010), preference for yeast metabolites phenylacetaldehyde and phenylacetic acid via IR84a (Grosjean et al., 2011), and attraction to ammonia and specific amines via IR92a (Min et al., 2013).

Taken together, these studies suggest that odorants linked to important ecological traits of insects are detected by receptors that have evolved a high degree of ligand specificity (**Figures 1A**, **2A**). These include receptors for sex- and host attractants as well as repellents, such as compounds produced by toxic microorganisms. This specificity minimizes cross-activation of receptors for ecologically relevant compounds by other non-relevant ones. Maintaining the high specificity of these receptors makes sense since mutations that reduce the specificity would most likely also reduce the fidelity of corresponding neuronal channels, increasing the risk of, for instance, mating- and oviposition mistakes, or death through inability to specifically detect toxins.

Evolution of Olfactory Receptors and Odor Tuning

Variation in Odorant Receptor Tuning Width

The increasing number of characterized receptors showing narrow tuning raises the possibility that most or even all ORs are specific, with what seems to be broadly tuned receptors only appearing so because key ligands have been missing from test panels or high screening concentrations were used. Despite this, there is still variation in OR tuning widths, even among the most thoroughly characterized ORs, with not all being as specific as the geosmin receptor in *Drosophila* (Stensmyr et al., 2012). In addition, it is possible that previously identified broadly tuned receptors (Hallem and Carlson, 2006; Carey et al., 2010) will remain broadly tuned even if tested at lower stimulus doses and expanded odor panels. Thus, an alternative scenario is that insect chemosensory systems contain both narrowly and broadly tuned receptors, providing the basis for both specific dedicated channels for certain compounds and combinatorial coding for others. If the insect olfactory system is indeed a hybrid between dedicated channels and combinatorial coding, the question becomes whether dedicated channels have evolved from a combinatorial coding system, or if the opposite is more likely. To address this question we should first discuss how it is thought the receptor families and the receptors themselves evolve at the molecular level.

Birth-and-Death Evolution of Receptor Families and the Ancestral State of Odor Coding

It is currently thought that the rapidly diversifying chemoreceptor gene families evolve according to a

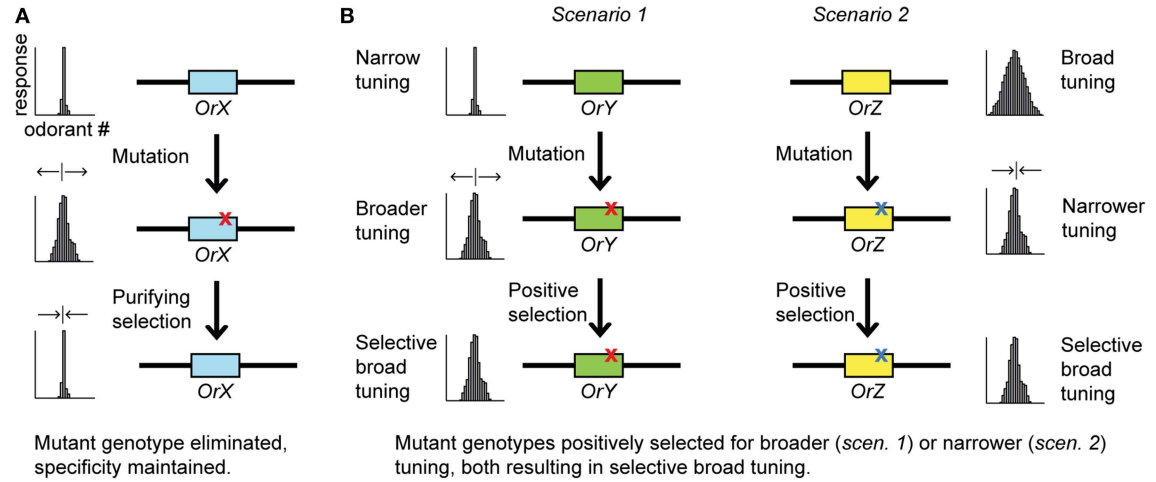

FIGURE 2 | (A) Odorant receptor *(Or)* genes coding for receptors that detect odorants important for fitness are generally under purifying selection and often narrowly tuned. Mutations to such *Ors* that change the response specificity will typically be selected against and ultimately eliminated from the population. **(B)** Selection may favor "selective broad tuning" that allows an OR to detect multiple odorants, such as those emmanating from the same ecological source. Two possible evolutionary scenarios are illustrated. Mutations that widen the response of a narrowly tuned OR (*scenario 1*), and mutations to a promisquous ORs that narrows the specificity to a certain group of odorants, are positively selected (*scenario 2*) with the same evolutionary "outcome". The tuning curves represent crude examples of different tuning widths and, as such, do not imply full coverage of chemical space.

birth-and-death evolutionary model, in which gene duplication events represent the births, and pseudogenization and deletion events the deaths (**Figure 3**) (Nei et al., 2008; Sánchez-Gracia et al., 2009; Ramdya and Benton, 2010; Cande et al., 2013). Within different insect lineages, various groups of receptor genes have expanded, whereas others have contracted or are simply no longer present within a particular insect group. The exception to this pattern is the conserved antennal IRs where orthologs of most genes are found in all insects (Croset et al., 2010), as well as the obligate OR co-receptor, Orco (originally Or83b in *D. melanogaster*), which is highly conserved among winged insects (Vosshall and Hansson, 2011; Missbach et al., 2014).

Consistent with the birth-and-death model of chemoreceptor evolution, all the receptors within a family will share a common ancestral gene. Thus, under this model, combinatorial coding cannot represent the ancestral state of olfactory coding, unless the appearance of the first receptor was accompanied by one or several duplication events (combinatorial coding requires at least two receptors). Combinatorial coding also requires more sophisticated neuronal wiring and processing, and it therefore seems more parsimonious that the olfactory system started off as simple. But what about the tuning width of the ancestral receptor(s)?

The first receptor with the ability to detect external volatiles had most likely not been pre-selected to bind any particular environmental odor cue. However, once it had gained a role as an odorant detector, its tuning might have evolved toward higher specificity for compounds such as certain mate cues or toxins. Alternatively, selection might have favored a broader response allowing detection of a larger range of general odorants using a single receptor. Can anything be learned from the recent functional studies of OSNs in basal insects? The most ancient family of insect chemoreceptors, the IRs (Croset et al., 2010), are present in primitive insects such as bristletails, silverfish and firebrats, which lack ORs with the exception of Orco in the firebrat (Missbach et al., 2014). Electrophysiological recordings from OSNs expressing IRs in the bristletail *Lepismachilis y-signata* using 36 stimuli at a high concentration (10^{-2} dilutions) showed a broader tuning as compared to the response of the IRs in *D. melanogaster* (Missbach et al., 2014). A similar result was obtained from the firebrat *Thermobia domestica*. Although these results suggest that primitive insects that lack ORs have olfactory IRs with broader tuning compared with insects that have both IRs and ORs, it is unknown whether this reflects the ancestral state or if the broad responses have evolved from IRs that were more selective hundreds of million years ago. The same reasoning applies to the receptors of the OR family, which are evolutionarily unrelated to the IR family. Missbach et al. (2014) identified 30 *Or* genes from transcriptomes of antennae and palps, and 23 functional sensillum types in the primitive neopteran leaf insect *Phyllium siccifolium*. Response profiles ranged from narrow to broad. However, the presence of 30 ORs suggests that the OR repertoire has undergone extensive evolution since the appearance of the first OR in Neoptera. Thus, today's response profiles might not inform us about the ancestral state.

Consistent with the birth-and-death model of OR evolution, all extant insect groups possess expanded OR repertoires. With a repertoire of receptors with different ligand specificities at hand, evolution can narrow down the response profile of a broadly tuned OR, but also broaden the response of a narrowly tuned OR (see also Sections Selection for Broad Receptor Tuning and Relaxed Purifying Selection Might Underlie Broad Receptor Tuning). Our understanding of the exact mutations that may

widen or narrow down the ligand selectivity of receptors is still in its infancy. However, a few recent studies have pinpointed certain amino acid sites as being crucial ligand determinants, thus providing molecular insight into the evolution of altered ligand affinity of odorant receptors.

Amino Acid Changes Affecting Receptor Tuning

Alterations of single amino acid residues (non-synonymous nucleotide mutations), or insertions and deletions, can significantly alter the ligand specificity of olfactory receptors (Pellegrino et al., 2011; Leary et al., 2012; Xu and Leal, 2013; Hughes et al., 2014). Mutations can occur within the major ligand binding site (orthosteric site), changing the response to include or preclude structurally similar compounds. Mutations can also occur at potential allosteric binding sites allowing or prohibiting binding of structurally dissimilar compounds (as discussed in Bohbot and Dickens, 2012). For instance, allosteric binding of fruit volatiles to the CO_2 receptor complex was implicated as a potential mechanism underlying reduced CO_2-mediated avoidance behavior in *Drosophila* in the presence of these odorants (Turner and Ray, 2009). In addition, mutations outside the ligand-binding site can also change receptor specificity and sensitivity, because they might change the gating equilibrium constant of the receptor (Colquhoun, 1998; Jadey et al., 2011; Hughes et al., 2014), or its structure and stability (Xu and Leal, 2013).

We are only just beginning to understand the positions and types of amino acid substitutions that may change the response of an OR. Mutations to amino acid 148 (located in transmembrane domain 3: TMD3) in OR3 of *Ostrinia nubilalis* and *O. furnacalis* have a large effect on the affinity to (*E*)-11-tetradecenyl acetate (E11), which is a pheromone component in *O. nubilalis*, but not in *O. furnacalis*. Wild type OR3 in *O. furnacalis* responds preferentially to (*E*)-12- and (*Z*)-12-tetradecenyl acetate. Changing amino acid residue 148 in *O. furnacalis* OR3 from threonine (wild type) to alanine (wild type in *O. nubilalis*) increased the sensitivity to E11 ca. 12-fold, thereby increasing the number of active ligands on this receptor. The converse mutation (A148T) to *O. nubilais* OR3 reduced the sensitivity to E11 to a similar extent (Leary et al., 2012). Similarly, a single amino acid substitution (V91A) in TMD2 of DmelOR59b, reflecting a natural polymorphism, significantly affected the response to the insect repellent DEET (Pellegrino et al., 2011). Also a few amino acids at the interface between extracellular loop 2 (ECL2) and TMD3 were implicated as determinants of odor sensitivity of DmelOR85b (Nichols and Luetje, 2010). In OR10 of mosquitoes, mutating either of three conserved proline residues in ECL2 to alanine lowered or abolished the response to skatole (Xu and Leal, 2013). The presence of neighboring glycine residues and nearby aromatic residues indicates that ECL2 contains multiple β-turns, and mutations to these residues also abolished the response. The presence of conserved β-turns also in moth ORs responding to structurally different odorants suggests that ECL2 acts as a lid to cover a membrane embedded binding pocket, rather than representing a specificity determinant of the binding pocket (Xu and Leal, 2013). Additionally, Hughes et al. (2014) identified 11 amino acid residues that affected the ligand specificities of *A. gambiae* OR13 and OR15. Mutations to alanine residue 195 at the interface between ECL2 and TMD4 had the most profound effect on the response to acetophenone, with the sensitivity being correlated to the amino acid side chain length. Together, these findings suggest that odorant receptors contain several amino acid positions located in both ECLs and TMDs in the TMD2-4 region that can be altered for tuning of odor specificity. Based on three-dimensional protein modeling, Hopf et al. (2015) suggest that this region that houses the ligand specificity determinants is likely to be a part of a ligand-binding site, however this needs experimental verification. With a multitude of sites and amino acid types at hand, it appears as if there is substantial opportunity for evolution to shape OR specificity, and in order to evolve a combinatorial odor coding system, broadly tuned receptors are required. We next examine how broad tuning can evolve through positive selection, as well as via neutral evolution under the umbrella of the birth-and-death evolutionary model.

Selection for Broad Receptor Tuning

Natural selection can favor a mutation that results in a more broadly tuned receptor (**Figure 2B**). It is important to note that this is not the same as selection for a combinatorial coding system *per se*, which, in addition to broadly tuned receptors, also requires overlap between different receptors' response spectra and potentially subsequent modulation of central processing mechanisms in the brain (Cande et al., 2013). For instance, if several structurally similar compounds emanate from the same or an equally "good" (or bad) source, a broader tuning allowing the OR to detect several of these compounds would be expected to increase the ability to detect the scent of such a source, which might be a host or non-host plant. Evolution of such "selective broad tuning" could also start from the other direction, i.e., mutations to a highly promiscuous receptor that restricts the affinity to include only compounds from the same ecological source might be favored by selection (**Figure 2B**). Either of these evolutionary pathways is a plausible scenario for how the dedicated neuronal circuit in *D. melanogaster* for citrus odors came to be activated by a somewhat broadly tuned receptor (Dweck et al., 2013). OR19a detects several terpenoid compounds of citrus fruits, but since these compounds are not abundant in other fruits that flies naturally encounter, they can all be used as a reliable signal from the preferred oviposition substrate. Similarly, the receptor DmelOR22a that responds to several fruit-related compounds (mainly esters) might have a selective broad tuning for the same reason. In addition, this particular OR was used to demonstrate that different ligands may compete for the same binding site (syntopic interactions) in a broadly tuned receptor, providing a means for mixture processing at the periphery (Münch et al., 2013). This may represent an additional advantage for a receptor to be broadly tuned.

Selective broad tuning of receptors for repellents might be favored by selection for the same reason. For instance, attraction to aggregation pheromones by a large number of conifer-feeding bark beetle species is antagonized by green leaf volatiles (GLV) (Zhang and Schlyter, 2004). The antagonizing GLVs are mostly C_6 alcohols, including 1-hexanol and monounsaturated analogs

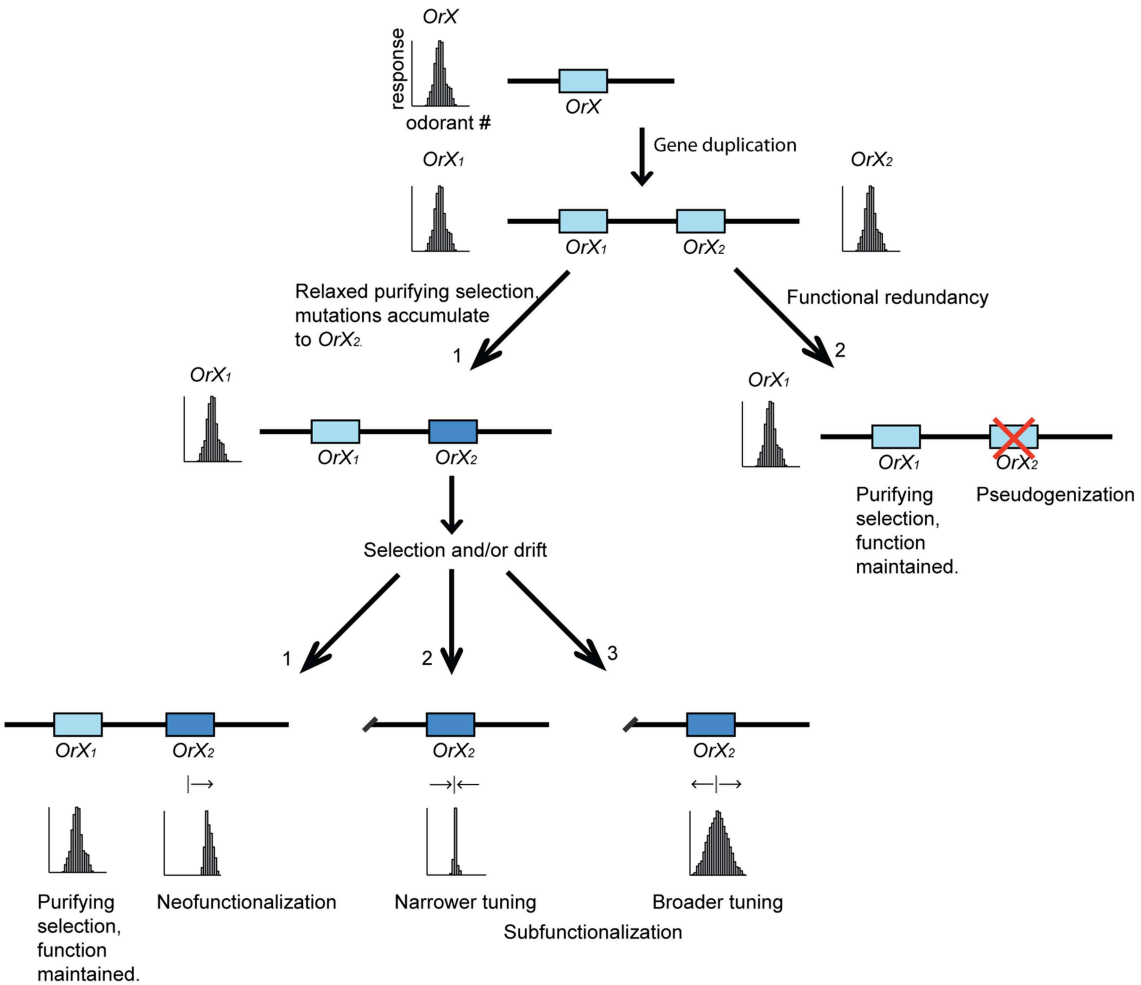

FIGURE 3 | The birth-and-death model of odorant receptor *(Or)* gene evolution. The hypothetical chain of events, in this example, starts with a gene duplication of *OrX* giving rise to two paralogous *Or* genes (OrX_1 and OrX_2). Frequently, one of the gene copies are pseudogenized due to functional redundancy, but both can also be maintained. In the latter case, mutations may accumulate to one of the genes due to relaxed purifying selection, given that the function of the other receptor remains intact. The mutated OR might acquire a novel function (neofunctionalization), or a similar function as its paralog (subfunctionalization). The tuning width can evolve by natural selection or genetic drift to become narrower or broader as compared to the parental receptor (*OrX*). The tuning curves represent crude examples of different tuning widths and, as such, do not imply full coverage of chemical space.

such as *E*2-, *E*3-, *Z*2-, and *Z*3-hexenol. Due to their abundance in green leaves of angiosperm plants and very low levels in conifers, GLVs are supposedly used by coniferous insect species as a trustworthy signal of unsuitable non-host breeding material. Electrophysiological recordings demonstrated that the European spruce bark beetle, *Ips typographus*, has a single OSN class that is highly sensitive but indiscriminate in its response to three different (1-hexanol, *E*2-, and *Z*3-hexenol) behaviorally antagonistic (and behaviorally redundant Unelius et al., 2014) GLV alcohols, and these compounds hardly activate any other OSN class (Andersson et al., 2009). This is in contrast to several species of angiosperm-feeding insects, which have several different OSN classes for GLVs, some specifically tuned to a single compound (Hansson et al., 1999; Larsson et al., 2001; Andersson et al., 2012a). Selective GLV receptors are likely to be important for species that feed on angiosperms, because specific ratios of these ubiquitous compounds can be utilized for host vs. non-host discrimination (Visser and Avé, 1978). However, different GLV compounds would have the same meaning to a conifer specialist, i.e., non-host. Thus, it is likely that broader tuning to detect several GLV compounds has been favored by natural selection in coniferous insect species.

Although most OSNs express only a single *Or* gene for odorant detection, there are exceptions to this canonical "one-OR-one-OSN rule" (Dobritsa et al., 2003; Hallem et al., 2004; Couto et al., 2005). Thus, another way of broadening the tuning of an OSN would be to co-express several *Or* genes. This option was recently shown in the European corn borer moth, *O. nubilalis*, where one OSN was found to co-express up to five pheromone receptor genes and respond broadly to several antagonistic compounds

(Koutroumpa et al., 2014). The authors hypothesized that this situation reflects the evolutionary pressure for being able to detect a wide range of heterospecific pheromone compounds to avoid non-specific mating - a mechanism contributing to pre-mating reproductive isolation. Similarly, Karner et al. (2015) found that 75 antennal OSNs in the malaria mosquito, *Anopheles gambiae,* co-express four *Or* genes (*AgamOr13*, *15*, *17*, and *55*) and about half of these OSNs also express *AgamOr16* and *AgamOr47*. Three of these ORs (AgamOR13, 15, and 16) have partly overlapping response spectra, including volatiles released by humans (Carey et al., 2010; Wang et al., 2010). It was therefore suggested that their co-expression mediates broad OSN tuning to improve the detection of complex host odor blends (Karner et al., 2015). These two recent examples suggest that co-expression of *Or* genes in the same neurons might be more common among insects than previously thought.

Relaxed Purifying Selection Might Underlie Broad Receptor Tuning

When a gene duplication event gives birth to a new *Or* gene, purifying selection is expected to be relaxed for one of the gene copies, given that the function of the other paralog is maintained (Ramdya and Benton, 2010). Frequently one of the copies will become pseudogenized due to functional redundancy (**Figure 3**). However, in some instances the second copy will be maintained due to this gene acquiring a new role (neofunctionalization), or sharing some of the role of the ancestral gene (subfunctionalization) or by gene conversion. These processes are thought to be important for the rapid diversification of chemoreceptor gene families (Nei et al., 2008).

Broad tuning of an olfactory receptor could be one of the possible outcomes of the relaxation of purifying selection (**Figure 3**). An example is found in the pheromone receptor OR7 from the leafroller moth, *Ctenopseustis herana*. This receptor is likely not required in sex pheromone communication in this species, but is capable of detecting a greater range of related pheromone components compared to its ortholog in *C. obliquana* that is specifically tuned to (*Z*)-8-tetradecenyl acetate and is required in pheromone communication (Steinwender et al., 2014). Broad tuning in this example is likely to be the derived state. Thus, broad tuning can in this case not be thought to be adaptive but rather a consequence of relaxed constraint. However, if the new detection capability of a broadly tuned receptor is beneficial, it might be maintained and positively selected, permitting the evolution of new signaling systems and ecological adaptations (Ramdya and Benton, 2010; Wyatt, 2014). In the case of pheromone receptors in moths, a number of species have been reported where at least one of the receptors displays broad tuning, detecting several of the sex pheromone components alongside receptors more selectively tuned to individual components (Miura et al., 2010; Zhang and Löfstedt, 2013). It would be interesting to investigate whether these broadly tuned pheromone receptors represent the ancestral or the derived state. In the turnip moth, *Agrotis segetum*, analysis of the selection pressures (i.e., the ratio of non-synonymous to synonymous mutations, dN/dS) acting on the pheromone receptors indicated that two of its broadly tuned receptors, AsegOR1 and AsegOR7, have evolved under relaxed constraint ($dN/dS = 1.02$, indicative of neutral evolution) (Zhang and Löfstedt, 2013). However, since the OR (AsegOR10) that roots the clade containing the two broadly tuned receptors was unresponsive to the seven test compounds, it is not possible to infer whether or not broad tuning is the derived state in this example (Zhang and Löfstedt, 2013).

More generally speaking, any mutation that changes the specificity of a pre-existing or newly born (and expressed) receptor will escape selection if the fitness of the individuals carrying the mutation is unaffected. For instance, if a mutation widens the response of a narrowly tuned receptor, fitness will not be affected if the insect in its natural environment never encounters the additional compounds that the receptor can pick up. In this scenario, the frequency of the mutated receptor allele in the population can be influenced randomly by genetic drift. The population might remain polymorphic at the mutated receptor locus, which is seen among ORs in natural populations of *Drosophila* (Rollmann et al., 2010; Pellegrino et al., 2011), but drift can also ultimately lead to fixation (or elimination) of the mutant receptor. If such a receptor is heterologously screened for responses to a large panel of ecologically relevant and irrelevant compounds, it might show a broad tuning, whereas in the natural environment it still acts as a specialist detector of high fidelity. Large-scale systematic screenings of ORs including both naturally occurring odorants and odorants that the insect is expected to never encounter are needed to test if this scenario is common in nature.

Having outlined evolutionary scenarios that may explain how some receptors can become broadly tuned, the question remains whether natural selection also could have acted to favor combinatorial coding in insects due to the associated benefits of this system?

Evolution of Combinatorial Coding in Insects

Advantages with Combinatorial Coding

While an olfactory system comprised solely of receptors for single compounds and dedicated neuronal pathways would provide high fidelity detection, it would also restrict the number of odorants that can be detected. In comparison, a lower level of fidelity is intuitively expected with a combinatorial coding system due to the overlapping responses of the receptors. However, there are also advantages associated with this system. Firstly, it renders the olfactory system more robust in response to disturbances. For instance, if the expression of a certain receptor gene fails or a sensillum is mechanically damaged, there would still be other receptors that respond to the compound, thus potentially rescuing the behavioral response (as implicated in Fishilevich et al., 2005; Keller and Vosshall, 2007). Secondly, broad receptor tuning increases the number of odorants and odor blends that can be detected by a receptor repertoire of a given size (Malnic et al., 1999; Bushdid et al., 2014), thus increasing perceived odor space and possibly allowing a higher degree of flexibility and

more olfactory behaviors to come about. Thus, the different advantages associated with the two contrasting modes of odor coding suggest that natural selection could have favored both, with either one of them dominating in certain species or olfactory sub-systems. We return to this in Section In which Insects can We Expect Combinatorial Coding to be the Dominant Coding 582 Strategy?, after addressing the neurodevelopmental constraints that possibly restrict the evolution of combinatorial coding in insects.

Neurological Constraints Acting Against the Evolution of Combinatorial Coding in Insects?

Though there might be advantages with a combinatorial system, it seems unlikely that natural selection originally acted in favor of combinatorial coding of single odorants in insects. This is because it would require "synchronized" mutations to multiple receptors in the periphery, alterations in regulatory genes controlling receptor gene expression (Ray et al., 2008), as well as potential modulation of neuronal circuits, structures, and processing mechanisms in the brain (Couto et al., 2005; Ramdya and Benton, 2010; Silbering et al., 2011; Cande et al., 2013). Rather, and as outlined above (Sections Selection for Broad Receptor Tuning and Relaxed Purifying Selection Might Underlie Broad Receptor Tuning), we believe it is more likely that the individual receptor genes respond to natural selection or relaxation of purifying selection, although an altered response profile of one receptor might change the selection pressures acting on another. Once receptors have acquired broad tuning, natural selection may secondarily start to mold a combinatorial coding system due to its associated benefits or, alternatively, to allow the animal to decipher the complex non-adaptive input from promiscuous receptors with overlapping ligand affinities (i.e., evolution builds on the system at hand).

However, it has been argued that constraints in developmental circuit patterning programs in insects might restrict their response to selection (Cande et al., 2013). For instance, glomerular development is fundamentally different between insects and mammals, and it is possible that neurological constraints in insects impede the evolution of combinatorial coding in this taxon compared to mammals. For instance, in mammals the ORs themselves guide axons to their glomeruli, which in simplified terms means that if a new OR is "born" a new glomerulus forms, and if an OR "dies" its glomerulus disappears (Mombaerts, 2006; Zou et al., 2009). In contrast, hard-wired genetic programs produce the insect AL, and the ORs play no role in glomerular formation (Imai et al., 2010; Ramdya and Benton, 2010). Likewise, the sensilla also develop under precise genetic control ensuring a stereotypical pattern of functional OSN classes across the sensory organs (Imai et al., 2010; Ramdya and Benton, 2010). Thus, insects might also require alterations in genetic control mechanisms to express new ORs (Wyatt, 2014). It is not known whether these constraints act against the evolution of combinatorial coding in insects, but intuitively it appears as if they are at least not facilitated. While the neurological constraints may be stronger in insects compared to mammals, nematodes are even more constrained with an extremely hard-wired nervous system. The nematode worm, *Caenorhabditis elegans*, has about 500 chemoreceptor genes, but only three pairs of neurons that detect volatile chemicals (Krieger and Breer, 1999; Troemel, 1999). Two of the neuron pairs induce attraction in response to activation of their many co-expressed receptors, and the other pair induces avoidance behavior (Troemel, 1999). This hard-wiring principally restricts the evolution of the olfactory sense to the chemoreceptors, leaving little room for combinatorial coding to evolve. Neurological constraints aside, broad receptor tuning provides the fundamental material for natural selection to start molding a combinatorial system. Due to the context-dependent advantages of this system, one might expect combinatorial coding to be more prevalent in certain insect groups than others.

In Which Insects can We Expect Combinatorial Coding to be the Dominant Coding Strategy?

One obvious advantage of broadly tuned receptors and a combinatorial coding system is the increase in the number of odorants that can be detected, thereby increasing perceived odor space. In insects, one could envision that being able to perceive a large odor space would be especially important for polyphagous species, those that have highly evolved semiochemical communication systems (e.g., social hymenopterans and termites), and for good learners that benefit from a greater flexibility in olfactory guided behaviors (e.g., honeybees and ants). For instance, honeybees have a remarkable ability to discriminate and learn hundreds of complex odor mixtures (Laska et al., 1999), and they also communicate using a large variety of pheromonal compounds. Early calcium imaging studies of honeybee AL activity suggested a combinatorial activation of glomeruli in response to high compound doses (undiluted compounds and 10^{-2} dilutions) (Joerges et al., 1997; Sachse et al., 1999). Similarly, recent recordings from honeybee projection neurons showed that all the 27 pheromonal compounds that were tested (including queen-, brood-, alarm-, and aggregation compounds) elicit a combinatorial activation pattern (Carcaud et al., 2015). In this study, compounds were tested either undiluted or at 50 μg/μl dilutions in isopropanol (5 μl loads in both cases). However, whether the observed combinatorial activation pattern is reflected in the response profiles of the ORs at lower odor concentrations is unknown because functional studies on honeybee ORs are largely lacking. In addition, little insight can be gained from single sensillum recordings from bees because their sensilla contain too many OSNs to be able to discern them based on spike amplitudes (Getz and Akers, 1994). Only a few hymenopteran ORs have been functionally described in heterologous systems. In the honeybee, the queen substance 9-oxo-2-decenoic acid is detected by a highly specific OR, AmelOR11 (Wanner et al., 2007). Moreover, AmelOR151 responds primarily to linalool, and AmelOR152 to a small set of other floral compounds (Claudianos et al., 2014). However, since the test odor panel in the latter study was modest (14 compounds), tuning widths are difficult to conclude. Functional studies of ORs in ants are also too scarce to conclude if ants mostly employ combinatorial coding or dedicated channels. To our knowledge only two ant ORs have been characterized. *Camponotus floridanus* OR263 responds specifically to 2,4,5-trimethylthiazole and *Harpegnathos saltator*

OR55 to 4-methoxyphenylacetone, both naturally occurring odorants (Zhou et al., 2012).

In addition, hymenopteran genomes contain larger numbers of *Or* genes than genomes from other insect orders, with for example honeybees having about 170 *Or* genes and some ant species over 400 (Robertson and Wanner, 2006; Robertson et al., 2010; Zhou et al., 2012). It is possible that this could facilitate the evolution of a combinatorial coding system, because a larger number of ORs increases the probability that the responses of their many ORs overlap. Alternatively, expressing many ORs might allow for a larger number of dedicated olfactory pathways without too much compromise of perceived odor space. In line with the latter reasoning are the results from Behrens et al.'s (2014) study on the response profiles of avian and amphibian bitter taste receptors (Tas2Rs) across a wide range of receptor repertoire sizes (2-ca. 50). The Tas2R receptors were always broadly tuned in species with two receptors, whereas individual receptors generally were more ligand-specific in species with a larger number of receptors.

So although the recordings from the honeybee brain suggest that combinatorial coding is operating in this species at high stimulus doses, there remains too little evidence to conclude whether or not this coding strategy would be more prevalent in polyphagous species, social insects and good learners, or species with large OR repertoires. This is, however, mainly due to lack of functional characterization of the ORs of such species. Studies should include a large proportion of the ORs encoded by the genome as well as a large set of ecologically relevant stimuli at natural concentrations in combination with measurements of glomerular activation in the AL.

In stark contrast to the hymenopterans, psyllid (Sternorrhyncha: Psyllidae) species are equipped with a truly minimalistic olfactory system. In three species studied using single sensillum electrophysiology, as few as 12 OSNs, grouped into four sensilla, appear to be devoted to plant odor detection (Kristoffersen et al., 2008b; Yuvaraj et al., 2013; Coutinho-Abreu et al., 2014), and these OSNs project to atypical, aglomerular, ALs (Kristoffersen et al., 2008a). Is there evidence of combinatorial coding in these species or do they mostly use narrowly tuned receptors? In fact, it appears as if the OSNs of psyllids are either very narrowly tuned or quite broadly tuned. Thus, the tuning ranges show a bimodal distribution and not a continuum as seen in most other insect species, at least at high stimulus concentrations (10^{-2} dilutions) (Coutinho-Abreu et al., 2014). This might indicate that the olfactory system of psyllids is hybrid between dedicated channels and combinatorial coding, possibly as a means of increasing odor space while retaining specificity for certain important odor cues. It remains to be seen whether or not this holds true also when challenged with larger odor panels and lower stimulus doses.

Conclusions and Future Directions

The established theory of combinatorial odor coding in insects has become challenged by several studies showing that ecologically relevant compounds are detected by highly specific receptors activating dedicated olfactory circuits. This has now been demonstrated for more than a third of the ORs encoded by the *D. melanogaster* genome, and for some ORs from other insect species and other classes of insect chemosensory receptors. The conclusion of combinatorial activation of broadly tuned receptors that was arrived at from earlier studies might be partly explained by experimental issues such as the use of high stimuli concentrations, the lack of ecologically relevant ligands in the screen, or the lack of quantification of airborne stimuli amounts.

We suggest that insect chemosensory systems are hybrids between combinatorial coding and dedicated circuits, with combinatorial coding likely evolving from dedicated circuits. The birth-and-death model of chemoreceptor evolution, including both adaptive and neutral changes, could allow some receptors and olfactory sub-systems to become broadly tuned, resulting in hybrid insect olfactory systems. In some cases, natural selection might have acted to favor broader tuning perhaps to increase the perceivable odor space, while in other instances, relaxed constraint might have allowed mutations to accumulate in receptors. Irrespective of the underlying evolutionary scenarios, once a subset of broadly tuned receptors is present, neurological or developmental mechanisms to process the more complex odor input might evolve in response to natural selection due to the associated benefits of combinatorial coding.

In different insects, one or the other system (dedicated vs. combinatorial) may come to dominate based on ecological context. Further studies are required to test this hypothesis and look at the nature of coding systems in non-drosophilid insects. Such studies should also reveal whether there is any correlation between OR repertoire size and the proportion of broadly tuned receptors, and therefore the extent of combinatorial coding in different insect groups. Future studies should also reveal whether more of the currently described broadly tuned receptors are actually dedicated detectors for yet to be discovered specific ligands. Finally, it will be interesting to learn to what extent the mechanisms that provide olfactory plasticity, such as regulation via differential *Or* gene expression (Fox et al., 2001; Reinhard and Claudianos, 2012; Claudianos et al., 2014) and neuromodulatory hormones (Flecke and Stengl, 2009; Ignell et al., 2009; Root et al., 2011), are operating in systems dominated by dedicated circuits or combinatorial systems.

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References

Ai, M., Min, S., Grosjean, Y., Leblanc, C., Bell, R., Benton, R., et al. (2010). Acid sensing by the *Drosophila* olfactory system. *Nature* 468, 691–695. doi: 10.1038/nature09537

Almaas, T. J., Christensen, T. A., and Mustaparta, H. (1991). Chemical communication in heliothine moths. *J. Comp. Physiol. A* 169, 249–258. doi: 10.1007/BF00206989

Andersson, M. N., Larsson, M. C., and Schlyter, F. (2009). Specificity and redundancy in the olfactory system of the bark beetle *Ips typographus*: single-cell responses to ecologically relevant odors. *J. Insect Physiol.* 55, 556–567. doi: 10.1016/j.jinsphys.2009.01.018

Andersson, M. N., Larsson, M. C., Svensson, G. P., Birgersson, G., Rundlöf, M., Lundin, O., et al. (2012a). Characterization of olfactory sensory neurons in the white clover seed weevil, *Apion fulvipes* (Coleoptera: Apionidae). *J. Insect Physiol.* 58, 1325–1333. doi: 10.1016/j.jinsphys.2012.07.006

Andersson, M. N., Schlyter, F., Hill, S. R., and Dekker, T. (2012b). What reaches the antenna? How to calibrate odor flux and ligand–receptor affinities. *Chem. Senses* 37, 403–420. doi: 10.1093/chemse/bjs009

Behrens, M., Korsching, S., and Meyerhof, W. (2014). Tuning properties of avian and frog bitter taste receptors dynamically fit gene repertoire sizes. *Mol. Biol. Evol.* 31, 3216–3227. doi: 10.1093/molbev/msu254

Bengtsson, J. M., Gonzalez, F., Cattaneo, A. M., Montagné, N., Walker, W. B., Bengtsson, M., et al. (2014). A predicted sex pheromone receptor of codling moth *Cydia pomonella* detects the plant volatile pear ester. *Front. Ecol. Evol.* 2:33. doi: 10.3389/fevo.2014.00033

Binyameen, M., Jankuvova, J., Blaženec, M., Jakuš, R., Song, L., Schlyter, F., et al. (2014). Co-localization of insect olfactory sensory cells improves the discrimination of closely separated odour sources. *Funct. Ecol.* 28, 1216–1223. doi: 10.1111/1365-2435.12252

Bohbot, J. D., and Dickens, J. C. (2009). Characterization of an enantioselective odorant receptor in the yellow fever mosquito *Aedes aegypti*. *PLoS ONE* 4:e7032. doi: 10.1371/journal.pone.0007032

Bohbot, J. D., and Dickens, J. C. (2012). Selectivity of odorant receptors in insects. *Front. Cell. Neurosci.* 6:29. doi: 10.3389/fncel.2012.00029

Bruce, T. J. A., Wadhams, L. J., and Woodcock, C. M. (2005). Insect host location: a volatile situation. *Trends Plant Sci.* 10, 269–274. doi: 10.1016/j.tplants.2005.04.003

Bruce, T. J., and Pickett, J. A. (2011). Perception of plant volatile blends by herbivorous insects – finding the right mix. *Phytochemistry* 72, 1605–1611. doi: 10.1016/j.phytochem.2011.04.011

Bushdid, C., Magnasco, M. O., Vosshall, L. B., and Keller, A. (2014). Humans can discriminate more than 1 trillion olfactory stimuli. *Science* 343, 1370–1372. doi: 10.1126/science.1249168

Cande, J., Prud'homme, B., and Gompel, N. (2013). Smells like evolution: the role of chemoreceptor evolution in behavioral change. *Curr. Opin. Neurobiol.* 23, 152–158. doi: 10.1016/j.conb.2012.07.008

Carcaud, J., Giurfa, M., and Sandoz, J.-C. (2015). Differential combinatorial coding of pheromones in two olfactory subsystems of the honey bee brain. *J. Neurosci.* 35, 4157–4167. doi: 10.1523/JNEUROSCI.0734-14.2015

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. doi: 10.1038/nature08834

Christensen, T. A., Mustaparta, H., and Hilderbrand, J. G. (1989). Discrimination of sex pheromone blends in the olfactory system of the moth. *Chem. Senses* 14, 463–477. doi: 10.1093/chemse/14.3.463

Claudianos, C., Lim, T., Young, M., Yan, S., Cristino, A. S., Newcomb, R. D., et al. (2014). Odor memories regulate olfactory receptor expression in the periphery. *Eur. J. Neurosci.* 39, 1642–1654. doi: 10.1111/ejn.12539

Colquhoun, D. (1998). Binding, gating, affinity and efficacy: the interpretation of structure−activity relationships for agonists and of the effects of mutating receptors. *Br. J. Pharmacol.* 125, 923–947. doi: 10.1038/sj.bjp.0702164

Coutinho-Abreu, I. V., Mcinally, S., Forster, L., Luck, R., and Ray, A. (2014). Odor coding in a disease-transmitting herbivorous insect, the Asian Citrus psyllid. *Chem. Senses* 39, 539–549. doi: 10.1093/chemse/bju023

Couto, A., Alenius, M., and Dickson, B. J. (2005). Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Curr. Biol.* 15, 1535–1547. doi: 10.1016/j.cub.2005.07.034

Croset, V., Rytz, R., Cummins, S. F., Budd, A., Brawand, D., Kaessmann, H., et al. (2010). Ancient protostome origin of chemosensory ionotropic glutamate receptors and the evolution of insect taste and olfaction. *PLoS Genet.* 6:e1001064. doi: 10.1371/journal.pgen.1001064

Dobritsa, A. A., Van Der Goes Van Naters, W., Warr, C. G., Steinbrecht, R. A., and Carlson, J. R. (2003). Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron* 37, 827–841. doi: 10.1016/S0896-6273(03)00094-1

Dweck, H. K. M., Ebrahim, S. A. M., Farhan, A., Hansson, B. S., and Stensmyr, M. C. (2015). Olfactory proxy detection of dietary antioxidants in *Drosophila*. *Curr. Biol.* 25, 455–466. doi: 10.1016/j.cub.2014.11.062

Dweck, H. K. M., Ebrahim, S. A. M., Kromann, S., Bown, D., Hillbur, Y., Sachse, S., et al. (2013). Olfactory preference for egg laying on *Citrus* substrates in *Drosophila*. *Curr. Biol.* 23, 2472–2480. doi: 10.1016/j.cub.2013.10.047

Fishilevich, E., Domingos, A. I., Asahina, K., Naef, F., Vosshall, L. B., and Louis, M. (2005). Chemotaxis behavior mediated by single larval olfactory neurons in *Drosophila*. *Curr. Biol.* 15, 2086–2096. doi: 10.1016/j.cub.2005.11.016

Flecke, C., and Stengl, M. (2009). Octopamine and tyramine modulate pheromone-sensitive olfactory sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner. *J. Comp. Physiol. A* 195, 529–545. doi: 10.1007/s00359-009-0429-4

Fox, A. N., Pitts, R. J., Robertson, H. M., Carlson, J. R., and Zwiebel, L. J. (2001). Candidate odorant receptors from the malaria vector mosquito *Anopheles gambiae* and evidence of down-regulation in response to blood feeding. *Proc. Natl. Acad. Sci. U.S.A.* 98, 14693–14697. doi: 10.1073/pnas.261432998

Getz, W. M., and Akers, R. P. (1994). Honeybee olfactory sensilla behave as integrated processing units. *Behav. Neural Biol.* 61, 191–195. doi: 10.1016/S0163-1047(05)80075-5

Grosjean, Y., Rytz, R., Farine, J.-P., Abuin, L., Cortot, J., Jefferis, G. S., et al. (2011). An olfactory receptor for food-derived odours promotes male courtship in *Drosophila*. *Nature* 478, 236–240. doi: 10.1038/nature10428

Hallem, E. A., and Carlson, J. R. (2006). Coding of odors by a receptor repertoire. *Cell* 125, 143–160. doi: 10.1016/j.cell.2006.01.050

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. doi: 10.1016/j.cell.2004.05.012

Hansson, B. S., and Stensmyr, M. C. (2011). Evolution of insect olfaction. *Neuron* 72, 698–711. doi: 10.1016/j.neuron.2011.11.003

Hansson, B. S., Larsson, M. C., and Leal, W. S. (1999). Green leaf volatile-detecting olfactory receptor neurones display very high sensitivity and specificity in a scarab beetle. *Physiol. Entomol.* 24, 121–126. doi: 10.1046/j.1365-3032.1999.00121.x

Hansson, B. S., Löfstedt, C., Löfqvist, J., and Hallberg, E. (1986). Spatial arrangement of different types of pheromone-sensitive sensilla in a male moth. *Naturwissenschaften* 73, 269–270. doi: 10.1007/BF00367782

Hildebrand, J. G., and Shepherd, G. M. (1997). Mechanisms of olfactory discrimination: Converging evidence for common principles across phyla. *Annu. Rev. Neurosci.* 20, 595–631. doi: 10.1146/annurev.neuro.20.1.595

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. doi: 10.1038/ncomms7077

Hughes, D. T., Pelletier, J., Luetje, C. W., and Leal, W. S. (2010). Odorant receptor from the southern house mosquito narrowly tuned to the oviposition attractant skatole. *J. Chem. Ecol.* 36, 797–800. doi: 10.1007/s10886-010-9828-9

Hughes, D. T., Wang, G., Zwiebel, L. J., and Luetje, C. W. (2014). A determinant of odorant specificity is located at the extracellular loop 2-transmembrane domain 4 interface of an *Anopheles gambiae* odorant receptor subunit. *Chem. Senses* 39, 761–769. doi: 10.1093/chemse/bju048

Ignell, R., Root, C. M., Birse, R. T., Wang, J. W., Nässel, D. R., and Winther, Å. M. E. (2009). Presynaptic peptidergic modulation of olfactory receptor neurons in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 13070–13075. doi: 10.1073/pnas.0813004106

Imai, T., Sakano, H., and Vosshall, L. B. (2010). Topographic mapping—the olfactory system. *Cold Spring Harb. Perspect. Biol.* 2:a001776. doi: 10.1101/cshperspect.a001776

Jadey, S. V., Purohit, P., Bruhova, I., Gregg, T. M., and Auerbach, A. (2011). Design and control of acetylcholine receptor conformational change. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4328–4333. doi: 10.1073/pnas.1016617108

Jiang, X.-J., Guo, H., Di, C., Yu, S., Zhu, L., Huang, L.-Q., et al. (2014). Sequence similarity and functional comparisons of pheromone receptor orthologs in two closely related *Helicoverpa* species. *Insect Biochem. Mol. Biol.* 48, 63–74. doi: 10.1016/j.ibmb.2014.02.010

Joerges, J., Küttner, A., Galizia, C. G., and Menzel, R. (1997). Representations of odours and odour mixtures visualized in the honeybee brain. *Nature* 387, 285–288. doi: 10.1038/387285a0

Jones, W. D., Cayirlioglu, P., Kadow, I. G., and Vosshall, L. B. (2007). Two chemosensory receptors together mediate carbon dioxide detection in *Drosophila*. *Nature* 445, 86–90. doi: 10.1038/nature05466

Karner, T., Schneider, I., Schultze, A., Breer, H., and Krieger, J. (2015). Co-expression of six tightly clustered odorant receptor genes in the antenna of the malaria mosquito *Anopheles gambiae*. *Front. Ecol. Evol.* 3:26. doi: 10.3389/fevo.2015.00026

Keller, A., and Vosshall, L. B. (2007). Influence of odorant receptor repertoire on odor perception in humans and fruit flies. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5614–5619. doi: 10.1073/pnas.0605321104

Keller, A., Zhuang, H., Chi, Q., Vosshall, L. B., and Matsunami, H. (2007). Genetic variation in a human odorant receptor alters odour perception. *Nature* 449, 468–472. doi: 10.1038/nature06162

Koutroumpa, F. A., Kárpáti, Z., Monsempes, C., Hill, S. R., Hansson, B. S., Jacquin-Joly, E., et al. (2014). Shifts in sensory neuron identity parallel differences in pheromone preference in the European corn borer. *Front. Ecol. Evol.* 2:65. doi: 10.3389/fevo.2014.00065

Kreher, S. A., Kwon, J. Y., and Carlson, J. R. (2005). The molecular basis of odor coding in the *Drosophila* larva. *Neuron* 46, 445–456. doi: 10.1016/j.neuron.2005.04.007

Kreher, S. A., Mathew, D., Kim, J., and Carlson, J. R. (2008). Translation of sensory input into behavioral output via an olfactory system. *Neuron* 59, 110–124. doi: 10.1016/j.neuron.2008.06.010

Krieger, J., and Breer, H. (1999). Olfactory reception in invertebrates. *Science* 286, 720–723. doi: 10.1126/science.286.5440.720

Kristoffersen, L., Hansson, B. S., Anderbrant, O., and Larsson, M. C. (2008a). Aglomerular hemipteran antennal lobes—basic neuroanatomy of a small nose. *Chem. Senses* 33, 771–778. doi: 10.1093/chemse/bjn044

Kristoffersen, L., Larsson, M. C., and Anderbrant, O. (2008b). Functional characteristics of a tiny but specialized olfactory system: olfactory receptor neurons of carrot psyllids (Homoptera: Triozidae). *Chem. Senses* 33, 759–769. doi: 10.1093/chemse/bjn034

Kwon, J. Y., Dahanukar, A., Weiss, L. A., and Carlson, J. R. (2007). The molecular basis of CO2 reception in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3574–3578. doi: 10.1073/pnas.0700079104

Larsson, M. C., Leal, W. S., and Hansson, B. S. (2001). Olfactory receptor neurons detecting plant odours and male volatiles in *Anomala cuprea* beetles (Coleoptera: Scarabaeidae). *J. Insect Physiol.* 47, 1065–1076. doi: 10.1016/S0022-1910(01)00087-7

Laska, M., Galizia, C. G., Giurfa, M., and Menzel, R. (1999). Olfactory discrimination ability and odor structure–activity relationships in honeybees. *Chem. Senses* 24, 429–438. doi: 10.1093/chemse/24.4.429

Leary, G. P., Allen, J. E., Bunger, P. L., Luginbill, J. B., Linn, C. E., Macallister, I. E., et al. (2012). Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14081–14086. doi: 10.1073/pnas.1204661109

Liu, C., Liu, Y., Guo, M., Cao, D., Dong, S., and Wang, G. (2014). Narrow tuning of an odorant receptor to plant volatiles in *Spodoptera exigua* (Hübner). *Insect Mol. Biol.* 23, 487–496. doi: 10.1111/imb.12096

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*. *Curr. Biol.* 17, 1533–1544. doi: 10.1016/j.cub.2007.07.062

Malnic, B., Hirono, J., Sato, T., and Buck, L. B. (1999). Combinatorial receptor codes for odors. *Cell* 96, 713–723. doi: 10.1016/S0092-8674(00)80581-4

Mansourian, S., and Stensmyr, M. C. (2015). The chemical ecology of the fly. *Curr. Opin. Neurobiol.* 34, 95–102. doi: 10.1016/j.conb.2015.02.006

Mathew, D., Martelli, C., Kelley-Swift, E., Brusalis, C., Gershow, M., Samuel, A. D. T., et al. (2013). Functional diversity among sensory receptors in a *Drosophila* olfactory circuit. *Proc. Natl. Acad. Sci. U.S.A.* 110, E2134–E2143. doi: 10.1073/pnas.1306976110

Min, S., Ai, M., Shin, S. A., and Suh, G. S. B. (2013). Dedicated olfactory neurons mediating attraction behavior to ammonia and amines in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 110, E1321–E1329. doi: 10.1073/pnas.1215680110

Missbach, C., Dweck, H. K. M., Vogel, H., Vilcinskas, A., Stensmyr, M. C., Hansson, B. S., et al. (2014). Evolution of insect olfactory receptors. *eLife* 3:e02115. doi: 10.7554/eLife.02115

Mitchell, R. F., Hughes, D. T., Luetje, C. W., Millar, J. G., Soriano-Agatón, F., Hanks, L. M., et al. (2012). Sequencing and characterizing odorant receptors of the cerambycid beetle *Megacyllene caryae*. *Insect Biochem. Mol. Biol.* 42, 499–505. doi: 10.1016/j.ibmb.2012.03.007

Miura, N., Nakagawa, T., Touhara, K., and Ishikawa, Y. (2010). Broadly and narrowly tuned odorant receptors are involved in female sex pheromone reception in *Ostrinia* moths. *Insect Biochem. Mol. Biol.* 40, 64–73. doi: 10.1016/j.ibmb.2009.12.011

Mombaerts, P. (2006). Axonal wiring in the mouse olfactory system. *Annu. Rev. Cell Dev. Biol.* 22, 713–737. doi: 10.1146/annurev.cellbio.21.012804.093915

Montagné, N., Chertemps, T., Brigaud, I., François, A., François, M. C., De Fouchier, A., et al. (2012). Functional characterization of a sex pheromone receptor in the pest moth *Spodoptera littoralis* by heterologous expression in Drosophila. *Eur. J. Neurosci.* 36, 2588–2596. doi: 10.1111/j.1460-9568.2012.08183.x

Münch, D., Schmeichel, B., Silbering, A. F., and Galizia, C. G. (2013). Weaker ligands can dominate an odor blend due to syntopic interactions. *Chem. Senses* 38, 293–304. doi: 10.1093/chemse/bjs138

Mustaparta, H., Angst, M. E., and Lanier, G. N. (1979). Specialization of olfactory cells to insect and host produced volatiles in the bark beetle *Ips pini* (Say). *J. Chem. Ecol.* 5, 109–123. doi: 10.1007/BF00987692

Nara, K., Saraiva, L. R., Ye, X., and Buck, L. B. (2011). A large-scale analysis of odor coding in the olfactory epithelium. *J. Neurosci.* 31, 9179–9191. doi: 10.1523/JNEUROSCI.1282-11.2011

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. doi: 10.1038/nrg2480

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. doi: 10.1074/jbc.M109.058321

Pellegrino, M., Steinbach, N., Stensmyr, M. C., Hansson, B. S., and Vosshall, L. B. (2011). A natural polymorphism alters odour and DEET sensitivity in an insect odorant receptor. *Nature* 478, 511–514. doi: 10.1038/nature10438

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. doi: 10.1371/journal.pone.0010090

Pelz, D., Roeske, T., Syed, Z., De Bruyne, M., and Galizia, C. G. (2006). The molecular receptive range of an olfactory receptor *in vivo* (*Drosophila melanogaster* Or22a). *J. Neurobiol.* 66, 1544–1563. doi: 10.1002/neu.20333

Priesner, E. (1986). "Correlating sensory and behavioural responses in multichemical pheromone systems of Lepidoptera," in *Mechanisms in Insect Olfaction,* eds T. L. Payne, M. C. Birch and C. E. J. Kennedy (Oxford: Clarendon press), 225–241.

Ramdya, P., and Benton, R. (2010). Evolving olfactory systems on the fly. *Trends Genet.* 26, 307–316. doi: 10.1016/j.tig.2010.04.004

Ray, A., Van Naters, W. V. D. G., and Carlson, J. R. (2008). A regulatory code for neuron-specific odor receptor expression. *PLoS Biol.* 6:e125. doi: 10.1371/journal.pbio.0060125

Reinhard, J., and Claudianos, C. (2012). "Molecular insights into honey bee brain plasticity," in *Honeybee Neurobiology and Behavior,* eds C. G. Galizia, D. Eisenhardt and M. Giurfa. (New York, NY: Springer), 359–372.

Riffell, J. A., Shlizerman, E., Sanders, E., Abrell, L., Medina, B., Hinterwirth, A. J., et al. (2014). Flower discrimination by pollinators in a dynamic chemical environment. *Science* 344, 1515–1518. doi: 10.1126/science.1251041

Robertson, H. M., and Wanner, K. W. (2006). The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. *Genome Res.* 16, 1395–1403. doi: 10.1101/gr.5057506

Robertson, H. M., Gadau, J., and Wanner, K. W. (2010). The insect chemoreceptor superfamily of the parasitoid jewel wasp *Nasonia vitripennis*. *Insect Mol. Biol.* 19, 121–136. doi: 10.1111/j.1365-2583.2009.00979.x

Rollmann, S. M., Wang, P., Date, P., West, S. A., Mackay, T. F. C., and Anholt, R. R. H. (2010). Odorant receptor polymorphisms and natural variation in olfactory behavior in *Drosophila melanogaster*. *Genetics* 186, 687–697. doi: 10.1534/genetics.110.119446

Ronderos, D. S., Lin, C.-C., Potter, C. J., and Smith, D. P. (2014). Farnesol-detecting olfactory neurons in *Drosophila*. *J. Neurosci.* 34, 3959–3968. doi: 10.1523/JNEUROSCI.4582-13.2014

Root, C. M., Ko, K. I., Jafari, A., and Wang, J. W. (2011). Presynaptic facilitation by neuropeptide signaling mediates odor-driven food search. *Cell* 145, 133–144. doi: 10.1016/j.cell.2011.02.008

Sachse, S., Rappert, A., and Galizia, C. G. (1999). The spatial representation of chemical structures in the antennal lobe of honeybees: steps towards the olfactory code. *Eur. J. Neurosci.* 11, 3970–3982. doi: 10.1046/j.1460-9568.1999.00826.x

Sánchez-Gracia, A., Vieira, F. G., and Rozas, J. (2009). Molecular evolution of the major chemosensory gene families in insects. *Heredity* 103, 208–216. doi: 10.1038/hdy.2009.55

Semmelhack, J. L., and Wang, J. W. (2009). Select *Drosophila* glomeruli mediate innate olfactory attraction and aversion. *Nature* 459, 218–223. doi: 10.1038/nature07983

Shirasu, M., Yoshikawa, K., Takai, Y., Nakashima, A., Takeuchi, H., Sakano, H., et al. (2014). Olfactory receptor and neural pathway responsible for highly selective sensing of musk odors. *Neuron* 81, 165–178. doi: 10.1016/j.neuron.2013.10.021

Silbering, A. F., Rytz, R., Grosjean, Y., Abuin, L., Ramdya, P., Jefferis, G. S. X. E., et al. (2011). Complementary function and integrated wiring of the evolutionarily distinct *Drosophila* olfactory subsystems. *J. Neurosci.* 31, 13357–13375. doi: 10.1523/JNEUROSCI.2360-11.2011

Steinwender, B., Thrimawithana, A. H., Crowhurst, R. N., and Newcomb, R. D. (2014). Pheromone receptor evolution in the cryptic leafroller species, *Ctenopseustis obliquana* and *C. herana*. *J. Mol. Evol.* 80, 42–56. doi: 10.1007/s00239-014-9650-z

Stensmyr, M. C., Dweck, H. K. M., Farhan, A., Ibba, I., Strutz, A., Mukunda, L., et al. (2012). A conserved dedicated olfactory circuit for detecting harmful microbes in *Drosophila*. *Cell* 151, 1345–1357. doi: 10.1016/j.cell.2012.09.046

Stensmyr, M. C., Giordano, E., Balloi, A., Angioy, A.-M., and Hansson, B. S. (2003). Novel natural ligands for *Drosophila* olfactory receptor neurones. *J. Exp. Biol.* 206, 715–724. doi: 10.1242/jeb.00143

Thoma, M., Hansson, B. S., and Knaden, M. (2014). Compound valence is conserved in binary odor mixtures in *Drosophila melanogaster*. *J. Exp. Biol.* 217, 3645–3655. doi: 10.1242/jeb.106591

Tømmerås, B. Å. (1985). Specialization of the olfactory receptor cells in the bark beetle *Ips typographus* and its predator *Thanasimus formicarius* to bark beetle pheromones and host tree volatiles. *J. Comp. Physiol. A* 157, 335–342. doi: 10.1007/BF00618123

Touhara, K., and Vosshall, L. (2009). Sensing odorants and pheromones with chemosensory receptors. *Annu. Rev. Physiol.* 71, 307–332. doi: 10.1146/annurev.physiol.010908.163209

Troemel, E. R. (1999). Chemosensory signaling in *C. elegans*. *Bioessays* 21, 1011–1020.

Turner, S. L., and Ray, A. (2009). Modification of CO2 avoidance behaviour in *Drosophila* by inhibitory odorants. *Nature* 461, 277–281. doi: 10.1038/nature08295

Unelius, R. C., Schiebe, C., Bohman, B., Andersson, M. N., and Schlyter, F. (2014). Non-host volatile blend optimization for forest protection against the european spruce bark beetle, *Ips typographus*. *PLoS ONE* 9:e85381. doi: 10.1371/journal.pone.0085381

Visser, J. H., and Avé, D. A. (1978). General green leaf volatiles in the olfactory orientation of the Colorado beetle, *Leptinotarsa decemlineata*. *Entomol. Exp. Appl.* 24, 538–549. doi: 10.1111/j.1570-7458.1978.tb02838.x

Vosshall, L. B., and Hansson, B. S. (2011). A unified nomenclature system for the insect olfactory coreceptor. *Chem. Senses* 36, 497–498. doi: 10.1093/chemse/bjr022

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. U.S.A.* 107, 4418–4423. doi: 10.1073/pnas.0913392107

Wanner, K. W., Nichols, A. S., Allen, J. E., Bunger, P. L., Garczynski, S. F., Linn, C. E. Jr., et al. (2010). Sex pheromone receptor specificity in the European corn borer moth, *Ostrinia nubilalis*. *PLoS ONE* 5:e8685. doi: 10.1371/journal.pone.0008685

Wanner, K. W., Nichols, A. S., Walden, K. K. O., Brockmann, A., Luetje, C. W., and Robertson, H. M. (2007). A honey bee odorant receptor for the queen substance 9-oxo-2-decenoic acid. *Proc. Natl. Acad. Sci. U.S.A.* 104, 14383–14388. doi: 10.1073/pnas.0705459104

Wetzel, C. H., Oles, M., Wellerdieck, C., Kuczkowiak, M., Gisselmann, G., and Hatt, H. (1999). Specificity and sensitivity of a human olfactory receptor functionally expressed in human embryonic kidney 293 cells and *Xenopus laevis* Oocytes. *J. Neurosci.* 19, 7426–7433.

Wu, W., Anton, S., Löfstedt, C., and Hansson, B. S. (1996). Discrimination among pheromone component blends by interneurons in male antennal lobes of two populations of the turnip moth, *Agrotis segetum*. *Proc. Natl. Acad. Sci. U.S.A.* 93, 8022–8027. doi: 10.1073/pnas.93.15.8022

Wyatt, T. D. (2014). *Pheromones and Animal Behavior: Chemical Signals and Signatures*. New York, NY: Cambridge University Press.

Xia, Y., Wang, G., Buscariollo, D., Pitts, R. J., Wenger, H., and Zwiebel, L. J. (2008). The molecular and cellular basis of olfactory-driven behavior in *Anopheles gambiae* larvae. *Proc. Natl. Acad. Sci. U.S.A.* 105, 6433–6438. doi: 10.1073/pnas.0801007105

Xu, F., Greer, C. A., and Shepherd, G. M. (2000). Odor maps in the olfactory bulb. *J. Comp. Neurol.* 422, 489–495. doi: 10.1002/1096-9861(20000710)422:4<489::AID-CNE1>3.0.CO;2-#

Xu, P., and Leal, W. S. (2013). Probing insect odorant receptors with their cognate ligands: insights into structural features. *Biochem. Biophys. Res. Commun.* 435, 477–482. doi: 10.1016/j.bbrc.2013.05.015

Yuvaraj, J. K., Andersson, M. N., Steinbauer, M., Farnier, K., and Anderbrant, O. (2013). Specificity and sensitivity of plant odor-detecting olfactory sensory neurons in *Ctenarytaina eucalypti* (Sternorrhyncha: Psyllidae). *J. Insect Physiol.* 59, 542–551. doi: 10.1016/j.jinsphys.2013.03.004

Zhang, D.-D., and Löfstedt, C. (2013). Functional evolution of a multigene family: orthologous and paralogous pheromone receptor genes in the turnip moth, *Agrotis segetum*. *PLoS ONE* 8:e77345. doi: 10.1371/journal.pone.0077345

Zhang, Q.-H., and Schlyter, F. (2004). Olfactory recognition and behavioural avoidance of angiosperm nonhost volatiles by conifer-inhabiting bark beetles. *Agric. For. Entomol.* 6, 1–19. doi: 10.1111/j.1461-9555.2004.00202.x

Zhou, X., Slone, J. D., Rokas, A., Berger, S. L., Liebig, J., Ray, A., et al. (2012). Phylogenetic and transcriptomic analysis of chemosensory receptors in a pair of divergent ant species reveals sex-specific signatures of odor coding. *PLoS Genet.* 8:e1002930. doi: 10.1371/journal.pgen.1002930

Zou, D.-J., Chesler, A., and Firestein, S. (2009). How the olfactory bulb got its glomeruli: a just so story? *Nat. Rev. Neurosci.* 10, 611–618. doi: 10.1038/nrn2666

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Co-expression of six tightly clustered odorant receptor genes in the antenna of the malaria mosquito *Anopheles gambiae*

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The behavior of female malaria mosquitoes, *Anopheles gambiae*, especially seeking out blood hosts or selecting oviposition sites, highly depends on the detection of relevant odorants by their sense of smell. This is mediated by olfactory sensory neurons (OSNs) which express distinct odorant receptor (OR) types. In the genome of *A. gambiae* 76 genes have been annotated to encode putative odorant receptors and the majority of these AgOR genes are arranged in clusters. To assess whether clustered AgOR genes are expressed in a characteristic manner we explored the topographic expression pattern of six tightly adjoined AgOR genes in the female antenna. Whole mount fluorescence *in situ* hybridization experiments were performed to visualize the olfactory neurons which express a distinct AgOR type in order to determine the number and the distribution of the cells. We found that within the 13 antennal segments about 75 cells contain mRNA for the four receptor types AgOR13, AgOR15, AgOR17, and AgOR55. Moreover, about half of these cells also transcribe mRNA for the subtypes AgOR16 and AgOR47. Subsequent RT-PCR experiments with primer pairs spanning the coding regions of adjacent AgOR genes revealed the existence of polycistronic mRNA. This result indicates that individual genes were not transcribed but mRNA was comprised of coding sequence from several genes within the studied cluster. Taken together, the data indicate a unique principle for the expression of odorant receptor genes arranged in a large cluster and suggest that the corresponding olfactory neurons are endowed with a distinct set of odorant receptor types.

Keywords: insect olfaction, antenna, odorant receptor, expression, polycistronic mRNA

Introduction

Blood-sucking insects are dreaded for their capacity to transfer life-threatening diseases. The anthropophilic mosquito, *Anopheles gambiae,* is the main vector for the perilous malaria parasite, *Plasmodium falciparum,* affecting millions of people worldwide every year (WHO, 2013). Female malaria mosquitoes find human hosts, oviposition sites and nectar sources mainly by chemical cues recognized by their olfactory system (Takken and Knols, 1999; Zwiebel and Takken, 2004). This is accomplished by olfactory sensory neurons (OSNs) in hair-like sensilla on their antennae, as well as on their maxillary palps and the labellum (McIver, 1982; Kwon et al., 2006; Pitts and Zwiebel, 2006). The pivotal role of the antennae is underpinned by the number of OSNs;

female *A. gambiae* possess about 1500 OSNs per antenna, but only ∼200 OSNs per maxillary palp and ∼50 OSN per labellar lobe (Kwon et al., 2006; Qiu et al., 2006; Lu et al., 2007).

The responsiveness of OSNs is determined by the odorant receptors (ORs) in their dendritic membrane (Hallem et al., 2004; Jacquin-Joly and Merlin, 2004; Touhara and Vosshall, 2009; Hansson and Stensmyr, 2011; Guidobaldi et al., 2014). In addition, ionotropic receptors (IRs) and gustatory receptor types (GRs) mediate responses of OSNs to odorants and CO_2, respectively (Kwon et al., 2007; Benton et al., 2009; Rytz et al., 2013; Tauxe et al., 2013). In the genome of *A. gambiae* 76 genes have been identified which encode putative odorant receptors (AgORs) (Hill et al., 2002; Pitts et al., 2011), and for most of these genes, expression has been verified in olfactory appendices of adult mosquitoes or larvae (Fox et al., 2002; Iatrou and Biessmann, 2008; Pitts et al., 2011; Rinker et al., 2013). Functional expression of 50 AgOR types in *Xenopus* oocytes (Wang et al., 2010) or in the *Drosophila* empty neuron system (Carey et al., 2010) revealed characteristic ligand spectra for about 30 of the AgOR types.

Within the genome of *A. gambiae* the majority of AgOR genes are arranged in clusters which consist of two to 9 genes (http://metazoa.ensembl.org/Anopheles_gambiae) (Fox et al., 2002; Hill et al., 2002). Such a clustered genomic organization of genes that encode chemosensory receptors appears to be characteristic for insects; for example, it has also been reported for the mosquito *Aedes aegypti* (Bohbot et al., 2007), the beetle *Tribolium castaneum* (Engsontia et al., 2008) and the honey bee *Apis mellifera* (Robertson and Wanner, 2006). Typically such clusters comprise two or three OR genes, but much larger tandem arrays with up to 60 OR genes occur in the honey bee (Robertson and Wanner, 2006). It is largely unknown to what extent OR genes organized in a cluster underlie common control mechanisms which could result in a similar spatial expression pattern or a co-expression in the same cell. A co-expression of clustered OR genes in individual cells of *Drosophila melanogaster* is demonstrated by the presence of mRNAs for two OR-types within the same cells (Dobritsa et al., 2003; Couto et al., 2005; Ray et al., 2007). For *A. gambiae,* we have recently found that transcripts for the receptor types AgOR13 and AgOR55 co-exist in the same cells (Schultze et al., 2014). Interestingly, the genes encoding AgOR13 and AgOR55 are immediate neighbors within the largest AgOR gene cluster (hereafter named cluster1) in the genome of *A. gambiae*; it is comprised of a total of 9 AgOR genes (**Figure 1**). Within cluster1, the coding regions of OR genes are in the same orientation and are spaced by short stretches of DNA. The first three AgOR genes (*AgOR53*, *AgOR30* and *AgOR46*) are more separated from the six more downstream located receptor genes (*AgOR47*, *AgOR16*, *AgOR17*, *AgOR13*, *AgOR55,* and *AgOR15*); accordingly, they were designated as cluster1A and cluster1B, respectively (**Figure 1A**). In transcriptome analysis of female antennae rather low transcript levels were found for the three genes in cluster1A, whereas the expression levels for the six genes in cluster1B were generally quite high (Pitts et al., 2011; Rinker et al., 2013). Based on the similar levels of mRNA for the strikingly tightly clustered OR-genes in the genome, we hypothesized that the genes in cluster1B may underlie a common transcription control.

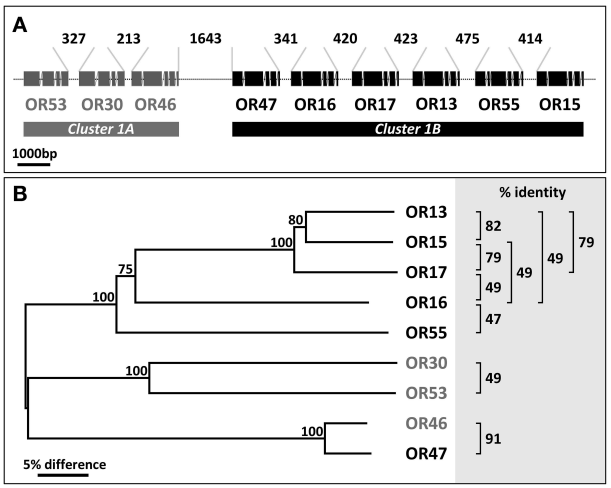

FIGURE 1 | Arrangement of cluster1 genes and relationship between AgORs. (A) Schematic representation of the chromosomal linkage and the intron/exon structure of AgOR genes in cluster1. All genes have the same orientation. The position and relative size of exons and introns were adopted from http://metazoa.ensembl.org/*Anopheles_gambiae* and drawn to scale. Exons represent protein coding regions only. The length of the intervening DNA strings connecting the exon regions of two adjacent AgORs (in base pairs, bp) is indicated. **(B**) Neighbor joining tree of cluster1 AgORs. The tree was constructed using MEGA5 based on a Clustal alignment of the AgOR amino acid sequences. Bootstrap support is based on 1000 replicates; support values >75% for branches are indicated. Sequence identities between pairs of AgORs which exceed 45% are given at the right.

Consequently, combinations of several or even all six AgOR types may be co-expressed in individual cells. Moreover, they may be translated from a large, polycistronic transcript, as reported for a large cluster of genes encoding gustatory receptors in the fruitfly (Slone et al., 2007). Here, we have used whole mount fluorescence *in situ* hybridization (WM-FISH) to explore the expression patterns of the six genomically adjoined AgOR types in the antenna of female *A. gambiae* and applied reverse transcription PCR (RT-PCR) to assess the presence of polycistronic mRNA encoding the receptor types.

Materials and Methods

Animal Rearing

Animals were reared to adults at 28°C with a day-night cycle of 12:12. Eggs and larvae of the *Anopheles gambiae* (Giles) s.s. strain RSP-H were kindly provided by Bayer CropScience, Monheim, Germany. The laboratory strain was originally derived from the region of Kisumu, Kenya and has been in rearing at Bayer since 2009 (to date: 46th generation). After emergence, animals had access to 10% sucrose *ad libitum*. For the experiments, one-to-eight-day old animals were used.

Preparation of *In Situ* Hybridization Probes

Digoxigenin (DIG)-labeled and biotin-labeled antisense RNAs for *in situ* hybridization were transcribed from linearized recombinant plasmids containing AgOR coding regions using

the components of the Roche T3/T7/SP6 RNA transcription/labeling system (Roche, Mannheim, Germany) and recommended protocols. To improve tissue penetration, the labeled antisense RNA probes were subsequently fragmented to an average length of about 200 or 800 nucleotides by incubation in carbonate buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3, pH 10.2) following the protocol of Angerer and Angerer (1992).

Transcription vectors (pBluescript SK II) carrying the coding sequences for AgOR55 and AgOR68, respectively, were kindly provided by Prof. Kostas Iatrou (NCSR "Demokritos," Athens, Greece). For AgOR13, AgOR15, AgOR16, AgOR17, and AgOR47, respectively, coding regions were PCR-amplified from cDNA of female *A. gambiae* heads and cloned into the pGem-T vector (Promega, Puchheim, Germany) using standard protocols. The identities of the AgOR sequences were verified by sequencing.

Head cDNA was prepared by dissecting heads from cold-anesthetized animals and collecting them in a tube cooled on liquid nitrogen. The tissue was crushed in liquid nitrogen using a small mortar and pestle and homogenized in Trizol reagent (Invitrogen). Total RNA was prepared from the Trizol homogenate and poly $(A)^+$ RNA was isolated from total RNA applying oligo $(dT)_{25}$ magnetic dynabeads (Dynal, Oslo, Norway) according to the suppliers protocols. Poly $(A)^+$ RNA from heads were transcribed into cDNA using the superscript III reverse transcriptase system (Invitrogen) with synthesis at 50°C for 50 min, followed by incubation for 15 min at 70°C.

Whole Mount Fluorescence *In Situ* Hybridization (WM-FISH)

Several receptor proteins encoded by the genes in cluster1 share relatively high sequence identity, higher than 45% (**Figure 1B**); however, with the other 67 AgOR-types of *A. gambiae*, sequence identity (10–40%) is rather low (not shown). Within the cluster, the identity between AgOR pairs is generally below 90%, a value that is considered critical with respect to a possible cross hybridization of probes for different AgORs. The exception is the receptor pair AgOR46 and AgOR47, which are 91% identical; however, since the transcript level of AgOR46 in female antennae is very low (Pitts et al., 2011), hybridization signals obtained with the AgOR47 probe most likely represent AgOR47-expressing cells.

Whole mount fluorescence *in situ* hybridizations (WM-FISH) with single or combinations of probes were performed as described previously (Schultze et al., 2013, 2014) with a few modifications. The antennae were dissected from the head and transferred directly to fixation solution (4% paraformaldehyde in 0.1 M $NaCO_3$, pH 9.5, 0.03% Triton X-100). After fixation for 20–24 h at 6°C the antennae were washed at room temperature for 1 min in PBS (phosphate-buffered saline = 145 mM NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.1) containing 0.03% Triton X-100. In a drop of this solution, each antenna was carefully squeezed several times at different positions along the longitudinal axis using fine forceps. This was followed by incubation at room temperature in 0.2 M HCl, 0.03% Triton X-100 for 10 min, two washes for 2 min each in PBS with 0.03% Triton X-100 and a treatment for 10 min with acetylation solution (25% acetic anhydride freshly added to a 0.1 M triethanolamine solution). Subsequently, the antennae were washed three times in PBS with 0.03% Triton X-100 for 3 min and prehybridized in WM-FISH solution (50% formamide, 5xSSC, 1xDenhardt's reagent, 50 μg/ml yeast RNA, 1% Tween 20, 0.1% Chaps, 5 mM EDTA, pH 8.0) at 55°C for 6 h. In WM-FISH experiments with single probes, this step was followed by incubation for at least 48 h at the same temperature in WM-FISH solution containing a DIG-labeled antisense RNA probe. Then the antennae were washed four times for 15 min each in 0.1xSSC, 0.03% Triton X-100 at 60°C. After treatment with 1% blocking reagent (Roche) in TBS (100 mM Tris, 150 mM NaCl, pH 7.5), 0.03% Triton X-100 for 5–6 h at 6°C, DIG-labeled probes were detected by incubation for at least 48 h with an anti-DIG alkaline phosphatase-conjugated antibody (Roche) diluted 1:500 in TBS, 0.03% Triton X-100 with 1% blocking reagent. After five washes for 10 min each in TBS with 0.05% Tween 20 at room temperature, antennae were incubated in the dark for 7–8 h with HNPP (2-hydroxy-3-naphtoic acid-2′-phenylanilide phosphate, Roche) 1:100 in DAP-buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 8.0) at 6°C to visualize hybridization of DIG-labeled probes.

In double WM-FISH experiments, simultaneous hybridization was performed with differentially labeled antisense RNA (DIG- and biotin-labeled, respectively) for two AgOR types. For concurrent detection of DIG- and biotin-labeled probes, the antennae were incubated after the posthybridization washes with the anti-DIG AP-conjugated antibody (Roche, diluted 1:500) and a streptavidin horse radish peroxidase-conjugate (1:100, TSA kit, Perkin Elmer) in TBS, 0.03% Triton X-100, 1% blocking reagent for at least 48 h at 6°C. This was followed by five 10 min washes in TBS, 0.05% Tween 20 at room temperature and incubation with HNPP (1:100 in DAP-buffer) for 7–8 h at 6°C in the dark to visualize the hybridization of DIG-labeled probes. Subsequently, the antennae were washed three times for 5 min in TBS, 0.05% Tween 20, followed by visualization of the biotin-labeled probes using the TSA / FITC development (TSA kit, Perking Elmer) and incubation for 17–18 h at 6°C in the dark. Finally, the antennae were washed three times for 5 min each in TBS with 0.05% Tween 20, briefly rinsed in PBS and mounted in Mowiol solution (10% polyvinylalcohol 4-88, 20% glycerol in PBS).

Analysis of Hybridized Antennae

After the WM-FISH treatment, antennae were analyzed on a Zeiss LSM510 Meta laser scanning microscope (Zeiss, Oberkochen, Germany). Confocal image stacks of the red and green fluorescence channel as well as the transmitted-light channel were taken from single antennal segments. Selected optical planes from image stacks were used to prepare figures, with the fluorescence channels and the transmitted-light channel overlaid or shown separately. Appropriate programs (MS PowerPoint, Adobe Photoshop) were used to arrange the figures and to adjust the brightness or contrast for uniform tone within a single figure.

To examine the distribution and number of AgOR-expressing cells along the 13 antennal segments, the labeled cells in a given antennal segment were counted under fluorescence microscope inspection. Due to breakage of antennae or damage from

squeezing, in many cases it was not possible to count the positive cells on all 13 segments of the hybridized antennae. Therefore, the number of different segments (i.e., antennae) that were analyzed to determine the average number of AgOR-expressing cells for distinct segments differ (Table S2).

Reverse Transcription PCR—Detection of Polycistronic mRNA

To investigate whether polycistronic mRNA transcripts encoding several cluster1B AgORs exist, we performed reverse transcription (RT) PCR experiments with cDNA from the head (with appendices) of female *A. gambiae* and primer pairs spanning the intergenic region of two adjacent AgOR genes. The sense primer was directed against a region located within the second half of the coding sequence of the first AgOR type, whereas the antisense primer matched within the first half of the coding sequence of the AgOR type, which follows downstream in the genome (**Figure 4**). Primer pairs spanned at least one intron region, thus allowing us to distinguish PCR products obtained from cDNA to that from PCR bands which may have resulted from amplification of genomic DNA. The positions of the primer pairs within the different cluster1B AgORs are indicated in **Figure 4**; their sequences are listed in Table S1. Standard PCR reactions were performed in a total volume of 50 µl using the High Fidelity PCR Enzyme Mix (Thermo/Fisher Scientific, Schwerte, Germany) and 1 µl of the head cDNA (prepared as described above). The reaction was started at 94°C for 1 min 40 s, followed by 20 cycles with 94°C for 30 s, 55°C for 40 s, and 72°C for 1 min 30 s. In each cycle the annealing temperature was decreased by 0.5°C. Then another 20 cycles at 45°C annealing temperature were conducted, followed by a last elongation step at 72°C for 7 min. PCR products were analyzed on agarose gels and visualized after staining under UV light.

Results

Expression of Cluster1B AgORs in the Antenna of Female *A. gambiae*

We analyzed the expression of six AgORs genes that belong to the largest AgOR cluster in the genome of *A. gambiae*. Using labeled riboprobes and the WM-FISH method, we visualized the cells that express the AgOR-types and determined their topographic distribution pattern throughout the female antenna (**Figure 2**). AgOR16 and AgOR47 are expressed in about the same number of cells within each of the studied antennal segments (**Figure 2A**). Moreover, for both receptor types, the distribution of the labeled cells along the 13 antennal segments was similar with the highest number of cells on antennal segments 4 and 5 and only in rare cases did labeled cells appear in segment 1 (**Figure 2B**). Evaluating the distribution of cells positive for the receptor types AgOR13, AgOR15, AgOR17, and AgOR55 revealed a pattern that was similar to that obtained for cells expressing AgOR16 and AgOR47 (**Figure 2B**). However, the number of labeled cells on each of the 13 antennal segments was twice as high as the number of cells for AgOR16 and AgOR47 (**Figures 2A,B**, Table S2). This 2:1 ratio was clearly reflected in the total number of labeled cells per antenna. A quantification of all labeled cells on the 13 antennal segments (Table S2) resulted in similar numbers across various receptor types; in detail we revealed 78 AgOR13-, 77 AgOR15-, 70 AgOR17-, and 76 AgOR55-cells per antenna as well as 37 AgOR16- and 36 AgOR47-cells per antenna.

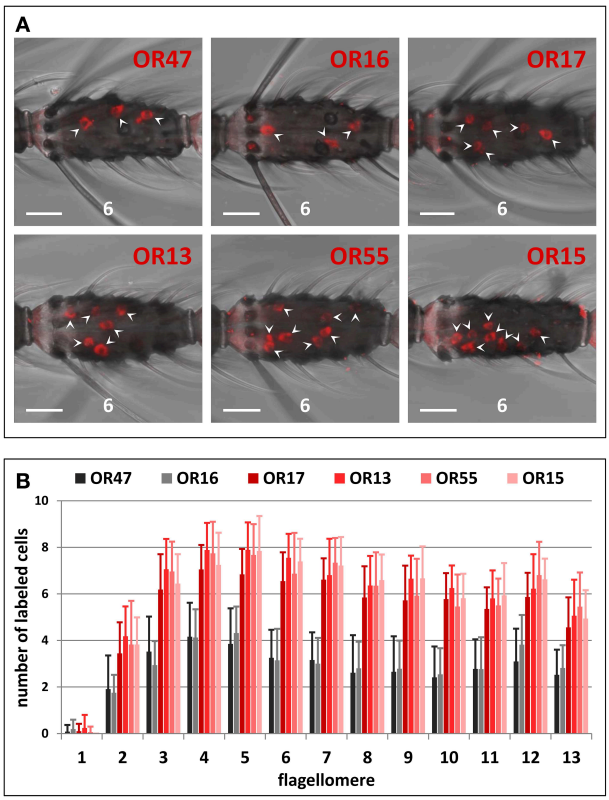

FIGURE 2 | Topography of cluster1B AgOR gene expression in the female antenna. (A) WM-FISH using DIG-labeled antisense RNA probes for the six cluster1B AgORs. Cells bearing AgOR transcripts have been visualized by a detection system leading to red fluorescence. The same (6th) flagellomere of the antenna from different animals is shown. Labeled cells are marked by arrowheads. Pictures have been arranged according to the order of the six AgORs in the genome (**Figure 1**). Probes for AgOR16 and AgOR47 label about half the number of cells compared to probes for AgOR13, AgOR15, AgOR16, and AgOR55, respectively. Scale bars: 20 µm. **(B)** Number and distribution of cells expressing cluster1B AgORs on the 13 flagellomeres of the female antenna. Segments are numbered 1–13 from proximal (head) to distal (tip of antenna). The average number of cells (± S.D.) shown for the different antennal segments has been determined by counting the cells from up to 28 antenna; details are given in Table S2.

Co-expression Pattern of Clustered AgORs

The similarities in the number and distribution of antennal cells which express the AgOR-subtypes encoded by genes arranged in cluster1B led us to suggest that these receptor types may be co-expressed by the same cells, or alternatively, may be expressed in adjacent cells of the same sensillum. In two-color WM-FISH experiments with combinations of receptor-specific probes (**Figure 3**) the two probes for the AgOR16/AgOR47 labeled the same cells (**Figure 3A**). This result indicates that AgOR16 and

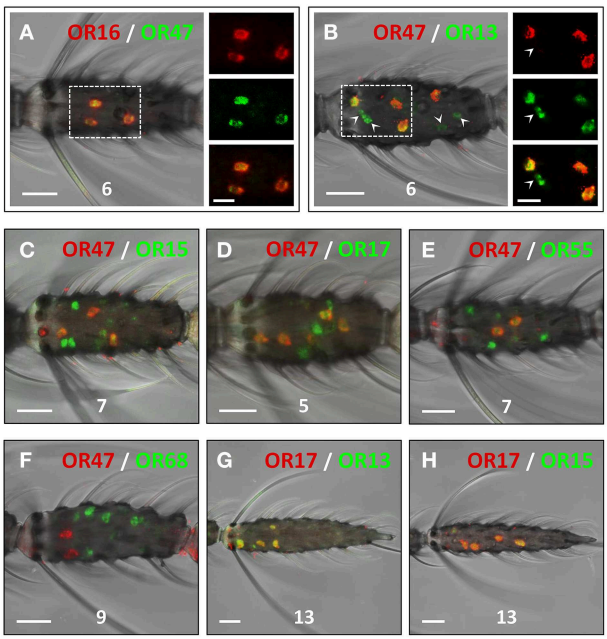

FIGURE 3 | Co-expression of AgORs in antennal cells. Double WM-FISH with female antennae using combinations of differentially labeled AgOR probes and visualization of cells bearing distinct AgOR transcripts by green (biotin) and red (DIG) fluorescence, respectively. Co-labeling of cells by both AgOR probes appears as yellow/orange color in the overlay of the red and green fluorescence channels. Numbers indicate the antennal segment shown. Scale bars: 20 μm. **(A,B)** WM-FISH using the combinations AgOR47/AgOR16 and AgOR47/AgOR13. The boxed areas are shown at higher magnification (right; scale bars: 10 μm), with the fluorescence channels displayed separately or superimposed. The AgOR47 and AgOR16 probes label the same cells **(A)**. AgOR47 labeled cells are also positive for the receptor types AgOR13 **(B)**, AgOR15 **(C)**, AgOR17 **(D)** and AgOR55 **(E)**, which in each case have transcripts in additional cells (green-labeled cells in **B–E**; arrowheads in **B**). **(F)** The probe combination AgOR47/AgOR68 (control) clearly labels different populations of cells. **(G,H)** The probe combinations AgOR17/AgOR13 and AgOR17/AgOR15 label the same cells.

AgOR47 are indeed co-expressed in the same cells and not in adjacent cells within a sensillum. Similar results were obtained in WM-FISH experiments with various combinations of probes for AgOR13, AgOR15, AgOR17, and AgOR55. Results are exemplarily shown for the combination AgOR13/AgOR17 (**Figure 3G**) and AgOR15/AgOR17 (**Figure 3H**). Each pair of the four AgOR probes revealed a 100% match of the red- and green-labeled cells (**Table 1**). These results led us to conclude that all four AgOR types are co-expressed in the same sensory neurons of the female antenna.

We next examined the relative spatial segregation of cells that co-express AgOR16 and AgOR47 vs. the cells that co-express the other four AgOR genes in cluster1B. In WM-FISH experiments with the AgOR47 probe in pairwise combination with probes for AgOR13, AgOR15, AgOR17, and AgOR55, for each pair a partial overlap of the labeled cells was found (**Figures 3B–E**, **Table 1**). This is shown in detail for the combination AgOR47/AgOR13 (**Figure 3B**); all AgOR47-positive cells (red) were also labeled by a probe for AgOR13 (green). In addition, several green-labeled

TABLE 1 | Co-expression pattern of cluster1B AgORs.

OR47					
C	**OR16**				
P	P	**OR17**			
P	P	C	**OR13**		
P	P	C	C	**OR55**	
P	P	C	C	C	**OR15**

Labeling patterns obtained in double WM-FISH experiments with female antennae and pairwise combinations of differentially labeled AgOR probes. C, complete overlap of labeled cells; P, partial overlap of labeled cells.

cells were found (**Figure 3B**, arrowheads, **Figures 3C–E**). In control experiments, application of a AgOR47 probe in combination with a biotin-labeled probe for the non-cluster1 receptor AgOR68 resulted in only clearly separated red- or green-labeled cells (**Figure 3F**), demonstrating that the two AgORs are expressed in different cell populations. Together, the results of the WM-FISH experiments indicate that a rather large number of sensory neurons (75 of about 1500 = 5%) in the female antenna co-transcribe the four cluster1B genes *AgOR13*, *AgOR15*, *AgOR17,* and *AgOR55*. Moreover, about half of these cells also comprise mRNA of AgOR16 and AgOR47 which is indicative for an expression of all six cluster1B AgORs in the cells.

Evidence for Polycistronic mRNA Encoding Cluster1B AgORs

The identification of transcripts for several AgOR genes of cluster1B in the same cells suggests that their transcription may be controlled by common regulatory mechanisms. A thorough assessment of the complete cluster1B sequence using the Eukaryotic Promotor Databases (EPD and EPDnew) (Dreos et al., 2013) did not lead to an identification of promotor motifs or common sequences in the 5′upstream regions of the six AgOR genes. In addition, the intervening sequences of two adjacent AgOR genes in cluster1B are very short (**Figure 1**). As a consequence of these observations, we hypothesized that the group of genes may be transcribed as polycistronic mRNA. To scrutinize this idea we performed RT-PCR experiments using five primer pairs each matching the coding regions of two adjacent AgOR genes and spanning the intervening regions OR47-16, OR16-17, OR17-13, OR13-55, and OR55-15 (**Figure 4**). In each case, the size of the PCR band corresponded well to the size expected if the exon sequences (but no introns) of the adjacent AgOR genes as well as the intervening regions are transcribed. Sequencing of the amplicons obtained with the primers for OR13-55 as well as for OR55-15 verified in both cases the exon sequences of the adjacent AgOR genes with a correct intron splicing. Moreover, the intervening sequences of the adjacent AgOR genes in the genome were found. From this region a stretch of 55 bp (of 475 bp) was missing for OR13-55 and 87 bp (of 414 bp) for the OR55-15 PCR products which most likely indicates a splicing of the precursor mRNA. Together the results demonstrate that individual genes in cluster1B are not transcribed but rather that polycistronic mRNA is generated encoding multiple AgORs.

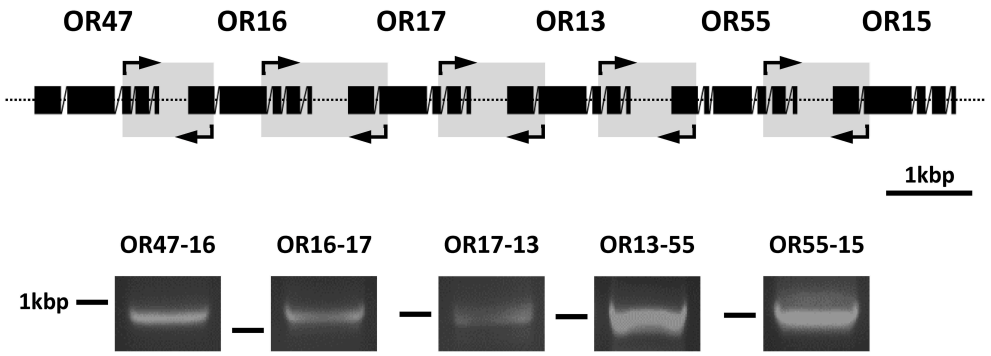

FIGURE 4 | Detection of polycistronic transcripts for cluster1B AgORs. Poly $(A)^+$ RNA prepared from the heads of female *A. gambiae* were reverse transcribed into cDNA and used in PCR reactions with primer pairs spanning parts of the coding regions of two adjacent AgORs as well as at least one intron and the region connecting the two AgOR genes being investigated. The position of the five sense and antisense primer pairs used are indicated by arrows framing gray boxes in the graphic representation of the exon-intron structure of the AgOR genes in cluster1B. Reverse transcription PCR products were analyzed on agarose gels. The obtained bands are shown below the region of the corresponding primer pair. The position of a 1 kbp marker band is indicated. Based on the primer design and assuming that the DNAs intervening the coding regions are transcribed, the predicted band sizes of the appropriately spliced cDNA products were: 938 bp for OR47-16, 1188 bp for OR16-17, 1030 bp for OR17-13, 992 bp for OR13-55, and 1027 bp for OR55-15.

Discussion

A close chromosomal linkage of odorant receptor genes is a common phenomenon in various insect species (Robertson and Wanner, 2006; Bohbot et al., 2007; Engsontia et al., 2008). However, very little is known about how the closely linked OR genes are transcribed and to what extent they may be co-expressed in the OSNs. The result of the present study indicates that in the malaria mosquito, *A. gambiae,* the transcription of six tightly clustered AgOR genes leads to polycistronic mRNA and to co-expression of several receptor types in OSNs in the female antenna.

This mode of gene expression is remarkable, since in eukaryotes the transcription of multiple genes as polycistronic mRNA is supposed to be quite rare, except for the nematode *Caenorhapditis elegans*, where around 15% of the genes are co-transcribed as operons (Blumenthal, 2004; Pi et al., 2009). For insect species, the first indication resulted from genome analysis of *Drosophila* species where, among the total of about 17,000 genes, around 100 dicistronic genes were predicted (Lin et al., 2007). In addition, polycistronic polypeptide coding RNAs (ppcRNAs) were identified in *Drosophila* and other insect species (Galindo et al., 2007). With respect to genes encoding chemosensory receptors of *D. melanogaster,* three cases of two genomically linked OR genes that are transcribed as dicistronic mRNA and co-expressed in OSNs have been reported (Ray et al., 2007). Surprisingly, a dicistronic transcript has been identified that underlies the co-expression of an odorant receptor type, DmOR10a, and a gustatory receptor type, DmGR10a, in the same cells (Fishilevich and Vosshall, 2005; Ray et al., 2007).

Interestingly, a case with similar features to the cluster1B genes of *A. gambiae* analyzed in this study was reported for six gustatory receptor genes of the fruitfly (Dahanukar et al., 2007; Slone et al., 2007). Similar to the six cluster1B odorant receptor genes, the genes for the sugar receptors DmGR64a – GR64f are tightly arranged and co-expressed in sensory neurons. Moreover, RT-PCR experiments indicated that the coding sequences of adjacent GR genes are on the same mRNA and that all six DmGR genes may be transcribed as a single polycistronic mRNA (Slone et al., 2007). Together, the actual data indicate co-expression of clustered chemosensory receptor genes in sensory neurons based on polycistronic mRNA. These recent findings suggest that co-expression of multiple receptor types from the same mRNAs transcript may be more widespread among insect species than previously thought.

For the co-transcription of the genes in cluster1B, the results of this study suggest two principles; it was found that one population of antennal OSNs contains transcripts for all six AgOR genes and another population has transcripts for four AgOR genes. This observation indicates that in the two cell populations different polycistronic mRNAs are transcribed from cluster1B. Based on the arrangement of genes in cluster1B, it is conceivable that one population of OSNs generates a polycistronic mRNA encoding AgOR17, AgOR13, AgOR55 and AgOR15, while the second population would generate a longer transcript, which also includes AgOR47 and AgOR16. Bioinformatic analyses of the sequences upstream and downstream of each of the cluster1B AgOR genes for promotor motifs and polyadenylation signals (AATAAA, ATTAAA) did not reveal any clue for explaining the transcription of mRNA comprising either four or six coding regions for AgORs, respectively. Thus, further investigation is needed to understand how differential transcription of the receptor genes in cluster1B in the two sensory neuron populations is controlled.

Our results show that a relatively high number of OSNs on a female antenna (about 5%) have transcripts from at least four cluster1B genes and half of these cells have transcripts from six genes. If all transcripts are in fact translated, the two populations of sensory neurons would be endowed with multiple receptor types. The high number of receptor types may render these cells

responsive to a broad spectrum of odorants. With respect to the ligand reactivity of these cells, functional analyses of some of the AgOR types encoded by genes in cluster1B in *Xenopus* oocytes and in the *Drosophila* empty neuron system have shown that the receptors AgOR13, AgOR15, and AgOR16 respond to distinct but partly overlapping spectra of ligands, which include volatiles found in human emanations (Carey et al., 2010; Wang et al., 2010).

Emanations from humans or oviposition sites consist of complex blends of various chemical compounds (Dormont et al., 2013; Himeidan et al., 2013). Thus, the first step to finding targets relevant to *A. gambiae* is to register a blend of volatile compounds with no need to discriminate distinct odorous compounds. Therefore, broadly tuned sensory neurons are particularly suitable for sensing a complex blend of components emitted from a relevant odor source. This scenario would be reminiscent of the bitter responsive cells in the taste buds of mice, which express many of the bitter receptor T2R types (Adler et al., 2000; Chandrashekar et al., 2000) making taste cells responsive to a large spectrum of potentially harmful compounds without discriminating among distinct molecules. Therefore, it is possible that antennal sensory neurons with multiple receptors may serve as "non-specific" sensors for odor blends and thus elicit the attention of female *A. gambiae* toward a relevant odor source and initiate directed behaviors, like host seeking or searching for oviposition sites. In this regard, targeting large gene clusters such as cluster1B of *A. gambiae* may be an interesting option for novel strategies toward a control of blood sucking mosquitoes and thereby reduce the danger of a transmission of life-threatening diseases.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fevo.2015.00026/abstract

References

Adler, E., Hoon, M. A., Mueller, K. L., Chandrashekar, J., Ryba, N. J., and Zuker, C. S. (2000). A novel family of mammalian taste receptors. *Cell* 100, 693–702. doi: 10.1016/S0092-8674(00)80705-9

Angerer, L. M., and Angerer, R. C. (1992). "*In situ* hybridization to cellular RNA with radiolabelled RNA probes," in *In Situ Hybridization,* ed D. G. Wilkinson (Oxford: IRL Press), 15–32.

Benton, R., Vannice, K. S., Gomez-Diaz, C., and Vosshall, L. B. (2009). Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. *Cell* 136, 149–162. doi: 10.1016/j.cell.2008.12.001

Blumenthal, T. (2004). Operons in eukaryotes. *Brief. Funct. Genomic. Proteomic.* 3, 199–211. doi: 10.1093/bfgp/3.3.199

Bohbot, J., 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. doi: 10.1111/j.1365-2583.2007.00748.x

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. doi: 10.1038/nature08834

Chandrashekar, J., Mueller, K. L., Hoon, M. A., Adler, E., Feng, L., Guo, W., et al. (2000). T2Rs function as bitter taste receptors. *Cell* 100, 703–711. doi: 10.1016/S0092-8674(00)80706-0

Couto, A., Alenius, M., and Dickson, B. J. (2005). Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Curr. Biol.* 15, 1535–1547. doi: 10.1016/j.cub.2005.07.034

Dahanukar, A., Lei, Y. T., Kwon, J. Y., and Carlson, J. R. (2007). Two Gr genes underlie sugar reception in *Drosophila*. *Neuron* 56, 503–516. doi: 10.1016/j.neuron.2007.10.024

Dobritsa, A. A., van der Goes van Naters, W., Warr, C. G., Steinbrecht, R. A., and Carlson, J. R. (2003). Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron* 37, 827–841. doi: 10.1016/S0896-6273(03)00094-1

Dormont, L., Bessiere, J. M., and Cohuet, A. (2013). Human skin volatiles: a review. *J. Chem. Ecol.* 39, 569–578. doi: 10.1007/s10886-013-0286-z

Dreos, R., Ambrosini, G., Cavin, P. R., and Bucher, P. (2013). EPD and EPDnew, high-quality promoter resources in the next-generation sequencing era. *Nucleic Acids Res.* 41, D157–D164. doi: 10.1093/nar/gks1233

Engsontia, P., Sanderson, A. P., Cobb, M., Walden, K. K., Robertson, H. M., and Brown, S. (2008). The red flour beetle's large nose: an expanded odorant receptor gene family in *Tribolium castaneum*. *Insect Biochem. Mol. Biol.* 38, 387–397. doi: 10.1016/j.ibmb.2007.10.005

Fishilevich, E., and Vosshall, L. B. (2005). Genetic and functional subdivision of the *Drosophila* antennal lobe. *Curr. Biol.* 15, 1548–1553. doi: 10.1016/j.cub.2005.07.066

Fox, A. N., Pitts, R. J., and Zwiebel, L. J. (2002). A cluster of candidate odorant receptors from the malaria vector mosquito, *Anopheles gambiae*. *Chem. Senses* 27, 453–459. doi: 10.1093/chemse/27.5.453

Galindo, M. I., Pueyo, J. I., Fouix, S., Bishop, S. A., and Couso, J. P. (2007). Peptides encoded by short ORFs control development and define a new eukaryotic gene family. *PLoS Biol.* 5:e106. doi: 10.1371/journal.pbio.0050106

Guidobaldi, F., May-Concha, I. J., and Guerenstein, P. G. (2014). Morphology and physiology of the olfactory system of blood-feeding insects. *J. Physiol. Paris* 108, 96–111. doi: 10.1016/j.jphysparis.2014.04.006

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. doi: 10.1016/j.cell.2004.05.012

Hansson, B. S., and Stensmyr, M. C. (2011). Evolution of insect olfaction. *Neuron* 72, 698–711. doi: 10.1016/j.neuron.2011.11.003

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. doi: 10.1126/science.1076196

Himeidan, Y. E., Temu, E. A., El rayah, E. A., Munga, S., and Kweka, E. J. (2013). Chemical cues for malaria vectors oviposition site selection: challenges and opportunities. *J. Insects* 2013:685182. doi: 10.1155/2013/685182

Iatrou, K., and Biessmann, H. (2008). Sex-biased expression of odorant receptors in antennae and palps of the African malaria vector *Anopheles gambiae*. *Insect Biochem. Mol. Biol.* 38, 268–274. doi: 10.1016/j.ibmb.2007.11.008

Jacquin-Joly, E., and Merlin, C. (2004). Insect olfactory receptors: contributions of molecular biology to chemical ecology. *J. Chem. Ecol.* 30, 2359–2397. doi: 10.1007/s10886-004-7941-3

Kwon, H. W., Lu, T., Rutzler, M., and Zwiebel, L. J. (2006). Olfactory responses in a gustatory organ of the malaria vector mosquito *Anopheles gambiae*. *Proc. Natl. Acad. Sci. U.S.A.* 103, 13526–13531. doi: 10.1073/pnas.0601107103

Kwon, J. Y., Dahanukar, A., Weiss, L. A., and Carlson, J. R. (2007). The molecular basis of CO_2 reception in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3574–3578. doi: 10.1073/pnas.0700079104

Lin, M. F., Carlson, J. W., Crosby, M. A., Matthews, B. B., Yu, C., Park, S., et al. (2007). Revisiting the protein-coding gene catalog of *Drosophila melanogaster* using 12 fly genomes. *Genome Res.* 17, 1823–1836. doi: 10.1101/gr.6679507

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*. *Curr. Biol.* 17, 1533–1544. doi: 10.1016/j.cub.2007.07.062

McIver, S. B. (1982). Sensilla of mosquitoes (Diptera: Culicidae). *J. Med. Entomol.* 19, 489–535.

Pi, H., Lee, L. W., and Lo, S. J. (2009). New insights into polycistronic transcripts in eukaryotes. *Chang Gung. Med. J.* 32, 494–498.

Pitts, R. J., Rinker, D. C., Jones, P. L., Rokas, A., and Zwiebel, L. J. (2011). Transcriptome profiling of chemosensory appendages in the malaria vector *Anopheles gambiae* reveals tissue- and sex-specific signatures of odor coding. *BMC Genomics* 12:271. doi: 10.1186/1471-2164-12-271

Pitts, R. J., and Zwiebel, L. J. (2006). Antennal sensilla of two female anopheline sibling species with differing host ranges. *Malaria J.* 5:26. doi: 10.1186/1475-2875-5-26

Qiu, Y. T., van Loon, J. J., Takken, W., Meijerink, J., and Smid, H. M. (2006). Olfactory coding in antennal neurons of the malaria mosquito, *Anopheles gambiae*. *Chem. Senses* 31, 845–863. doi: 10.1093/chemse/bjl027

Ray, A., van Naters, W. G., Shiraiwa, T., and Carlson, J. R. (2007). Mechanisms of odor receptor gene choice in *Drosophila*. *Neuron* 53, 353–369. doi: 10.1016/j.neuron.2006.12.010

Rinker, D. C., Pitts, R. J., Zhou, X., Suh, E., Rokas, A., and Zwiebel, L. J. (2013). Blood meal-induced changes to antennal transcriptome profiles reveal shifts in odor sensitivities in *Anopheles gambiae*. *Proc. Natl. Acad. Sci. U.S.A.* 110, 8260–8265. doi: 10.1073/pnas. 1302562110

Robertson, H. M., and Wanner, K. W. (2006). The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. *Genome Res.* 16, 1395–1403. doi: 10.1101/gr.5057506

Rytz, R., Croset, V., and Benton, R. (2013). Ionotropic receptors (IRs): chemosensory ionotropic glutamate receptors in *Drosophila* and beyond. *Insect Biochem. Mol. Biol.* 43, 888–897. doi: 10.1016/j.ibmb.2013.02.007

Schultze, A., Breer, H., and Krieger, J. (2014). The blunt trichoid sensillum of female mosquitoes, *Anopheles gambiae*: odorant binding protein and receptor types. *Int. J. Biol. Sci.* 10, 426–437. doi: 10.7150/ijbs.8754

Schultze, A., Pregitzer, P., Walter, M. F., Woods, D. F., Marinotti, O., Breer, H., et al. (2013). The co-expression pattern of odorant binding proteins and olfactory receptors identify distinct trichoid sensilla on the antenna of the malaria mosquito *Anopheles gambiae*. *PLoS ONE* 8:e69412. doi: 10.1371/journal.pone.0069412

Slone, J., Daniels, J., and Amrein, H. (2007). Sugar receptors in *Drosophila*. *Curr. Biol.* 17, 1809–1816. doi: 10.1016/j.cub.2007.09.027

Takken, W., and Knols, B. G. (1999). Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annu. Rev. Entomol.* 44, 131–157. doi: 10.1146/annurev.ento.44.1.131

Tauxe, G. M., MacWilliam, D., Boyle, S. M., Guda, T., and Ray, A. (2013). Targeting a dual detector of skin and CO2 to modify mosquito host seeking. *Cell* 155, 1365–1379. doi: 10.1016/j.cell.2013.11.013

Touhara, K., and Vosshall, L. B. (2009). Sensing odorants and pheromones with chemosensory receptors. *Annu. Rev. Physiol.* 71, 307–332. doi: 10.1146/annurev.physiol. 010908.163209

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. U.S.A.* 107, 4418–4423. doi: 10.1073/pnas.0913392107

WHO (2013). *World Malaria Report*. Geneva: WHO Press.

Zwiebel, L. J., and Takken, W. (2004). Olfactory regulation of mosquito-host interactions. *Insect Biochem. Mol. Biol.* 34, 645–652. doi: 10.1016/j.ibmb.2004.03.017

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Identification of candidate olfactory genes in *Leptinotarsa decemlineata* by antennal transcriptome analysis

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The sense of smell is critical for the survival of insects, by as insects detect odor signals in the environment and make appropriate behavioral responses such as host preference, mate choice, and oviposition site selection. The antenna is the main olfactory organ in insects. Multiple antennal proteins have been suggested to be involved in olfactory signal transduction pathway such as odorant receptors (ORs), ionotropic receptors (IRs), odorant binding proteins (OBPs), chemosensory proteins (CSPs) and sensory neuron membrane proteins (SNMPs). In this study, we identified several olfactory gene subfamilies in the economically important Coleopteran agricultural pest, *Leptinotarsa decemlineata*, by assembling the adult male and female antennal transcriptomes. In the male and female antennal transcriptome, we identified a total of 37 OR genes, 10 IR genes, 26 OBP genes, 15 CSP genes, and 3 SNMP genes. Further, expression of all candidate ORs was validated in male or female antenna by semi-quantitative reverse transcription PCR. Most of the candidate OR genes have similar expression levels in male and female. A few OR genes have been detected to have male-specific (*LdecOR6*) or male-biased (*LdecOR5*, *LdecOR12*, *LdecOR26,* and *LdecOR32*) expression. Additionally, two OR genes (*LdecOR3* and *LdecOR29*) were observed to be expressed higher in female. Our findings make it possible for future research of the olfactory system of *L. decemlineata* at the molecular level.

Keywords: transcriptome, olfactory gene, *Leptinotarsa decemlineata*, antenna, RT-PCR

Introduction

Olfaction, the sense of smell, is critically important for insects survival on earth through mediating key behaviors such as food identification, oviposition site selection, mate choice, predator avoidance, and so on (Mustaparta, 1990; Hildebrand, 1995; Sato and Touhara, 2009).

The antenna is the major organ for insect olfactory sensing and its surface is coved by thousands of special hair structures called "sensilla" (Hildebrand and Shepherd, 1997). The sensillum is where peripheral olfactory signal transduction events occur. Each sensillum contains the dendrites of olfactory receptor neurons (ORNs). And the axons of these ORNs are projected into the antennal lymph on toward the brain (Shanbhag et al., 1999, 2000). The ORNs act as biological transducers in that they convert the signal of ecologically relevant volatile chemicals into electrical

impulses. It has been shown that diverse olfactory genes are involved in different steps of this transduction process including odorant receptors (ORs), ionotropic receptors (IRs), odorant binding proteins (OBPs), chemosensory proteins (CSPs) and sensory neuron membrane proteins (SNMPs) (Rützler and Zwiebel, 2005; de Bruyne and Baker, 2008; Sato and Touhara, 2009). The signal transduction process can be summarized by the following steps: first, the hydrophobic chemical compounds encounter the sensilla and then enter into the sensillum lymph through the pores on the surface (Kanaujia and Kaissling, 1985; Kaissling and Colbow, 1987). Then, water-soluble OBPs/CSPs bind to the compounds and help them to translocate to the surface of ORNs (Pelosi and Maida, 1995; Foret et al., 2007; Laughlin et al., 2008; Zhou, 2010). The odorants finally activate the ORs/IRs expressed on the dendritic membrane of ORNs alone or in complex with the binding proteins (Wojtasek and Leal, 1999; Xu et al., 2005). SNMPs are thought to be expressed adjacent to ORs and are presumed to trigger ligand delivery to the receptor (Rogers et al., 2001; Benton et al., 2007; Vogt et al., 2009).

In this process, ORs play a central role as a bio-transducer, facilitating the conversion of the chemical message to an electrical signal. Although the ORs from both insects and vertebrate have seven transmembrane domains (TMDs), the insects ORs do not belong to the family of canonical G-protein coupled receptors (GPCRs), to which they have a reversed membrane topology (intracellular N-terminus) (Clyne et al., 1999; Benton et al., 2006). It is generally thought that each ORN expresses a conserved, OR co-receptor (Orco protein) and a divergent, conventional ORx, such that the heterodimer of Orco-OR forms an ion channel and mediates odorant-binding specificity (Larsson et al., 2004; Neuhaus et al., 2005; Sato et al., 2008; Wicher et al., 2008; Jones et al., 2011). In addition, an evolutionary ancient family of chemosensory receptors, the IRs, was recently identified in *Drosophila melanogaster* (Benton et al., 2009). IRs have structural similarity with ionotropic glutamate receptors, while they separate from each other in phylogenetic analysis (Benton et al., 2009; Croset et al., 2010). IRs are expressed largely by non-overlapping populations of ORNs and have been shown to be activated by a small odor panel that includes acetates and small amine-like volatile compounds (Abuin et al., 2011; Ai et al., 2013).

The study of insect olfactory genes, especially the ORs, was initially confounded on account of their extreme divergence, until olfactory genes were first comprehensively identified in *D. melanogaster* (Adams et al., 2000), and then in other insect species including *Anopheles gambiae* (Fox et al., 2001), *Bombyx mori* (Xia et al., 2004) and *Tribolium castaneum* (Richards et al., 2008) with the release of their genome sequences. The read length and output of next-generation sequencing continues to rise in recent years, meanwhile the cost has dramatically declined, but full genome sequencing of insects is still a challenge because of difficulty in assembling. The transcriptome sequencing approaches present an alternative advantage in olfactory gene identification in insect species where a genome sequence is not yet available. To date, insect antennal transcriptome sequencing has been successfully used to identify substantial numbers of candidate olfactory genes in *Manduca sexta* (Grosse-Wilde et al., 2011), *Helicoverpa armigera* (Liu et al., 2012), *Spodoptera littoralis* (Legeai et al., 2011; Jacquin-Joly et al., 2012; Poivet et al., 2013), *Chilo suppressalis* (Cao et al., 2014), *Cydia pomonella* (Bengtsson et al., 2012) etc. Most of these insects belong to the order Lepidoptera.

Coleopteran species constitute almost 25% of all known types of animal life-forms (Hunt et al., 2007). About 40% of all described insect species are beetles (about 400,000 species). In this, the largest insect order, olfactory genes have been identified from a few species: one from the genome of *T. castaneum* (Richards et al., 2008; Kim et al., 2010), and recently from the antennal transcriptomes of *Megacyllene caryae* (Mitchell et al., 2012), *Ips typographus* (Andersson et al., 2013), *Dendroctonus ponderosae* (Andersson et al., 2013), *Monochamus alternatus* (Wang et al., 2014), *Dastarcus helophoroides* (Wang et al., 2014), and *Rhyzopertha dominica* (Diakite et al., 2015). Thus, a greater effort must be made to investigate other beetle species in order to better understand the molecular biology of Coleopteran and insect olfaction.

The Colorado potato beetle *Leptinotarsa decemlineata* is a global crop pest, and it causes huge economic loss annually (Kuhar et al., 2006). The male-produced aggregation pheromone of this beetle has been identified (Dickens et al., 2002), but the molecular mechanisms of olfactory recognition in this insect is still unknown. In this study, we performed Illumina HiSeq 2000 sequencing of the transcriptome of adult male and female antennae of this important agricultural pest. Our goals were to identify olfaction-related genes and olfactory signal transduction mechanisms in this insect. Here we report the identification of 37 candidate OR genes, 10 IR genes, 26 OBP genes, 15 CSP genes, and 3 SNMP genes in the antennal transcriptome of *L. decemlineata*.

Methods

Insects, Dissection, and RNA Extraction

The *L. decemlineata* adults were collected from potato fields in Xinjiang Province, China. Male and female adults were separated, not considering the ages or mating status. The antennae were pulled off with tweezers grasped at the very root of the antennae. The separated antennae were stored in RNAlater (Ambion, Austin, TX, USA) and taken to the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing. After removing the residual RNAlater, the stored antennae were crushed with a vitreous homogenizer. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The RNA was dissolved in RNase-free water and the integrity and quantity of RNA was determined by gel electrophoresis and Nanodrop ND-2000 spectrophotometer (NanoDrop products, Wilmington, DE, USA). Residual gDNA in total RNA was removed by DNase I (Promega, Madison, WI, USA) before cDNA library construction.

cDNA Library Construction, Sequencing, and Assembly

Five micrograms of total RNA extracted from approximately 200 antennae of adult male or female adults were sent to Beijing Genome Institute (Shenzhen, China) for construction of cDNA library and sequencing. Briefly, mRNA was isolated and fragmented into 200–700 nt pieces. Random hexamers were used for first-strand cDNA synthesis. Then the second-strand cDNA was synthesized using RNase H and DNA polymerase I. The resulting double-stranded cDNAs were treated with T4 DNA Polymerase and T4 Polynucleotide Kinase for end-repairing and dA-tailing. After that, they were ligated to sequencing adaptors with barcode using T4 DNA ligase. Finally, fragments with around 200 bp length were collected by 2% agarose gel electrophoresis and purified with QiaQuick GelPurify Kit (Qiagen, Hilden, Germany), and used as templates for PCR amplification. The libraries were pair-end sequenced using PE90 strategy on Illumina HiSeq™ 2000 (Illumina, San Diego, CA, USA) at the Beijing Genome Institute. The male and female libraries were sequenced in one lane then raw-reads were sorted out by barcodes.

Raw reads from each library were filtered to remove low quality reads and the sequence reads containing adapters and poly-A/T tails. The resulting clean reads were assembled to produce unigenes with the short reads assembling program-Trinity using the default parameters (Grabherr et al., 2011). Then the unigenes from the two samples were pooled together and clustered by TGI Clustering Tool (TGICL) (Pertea et al., 2003). The consensus cluster sequences and singletons make up the unigenes dataset.

TABLE 1 | Assembly summary of *L. decemlineata* antennal transcriptome.

	Sample	Total number	Total length(nt)	Mean length(nt)	N50	Consensus sequences	Distinct clusters	Distinct singletons
Contig	Male	87,584	27,672,623	316	509	-	-	-
	Female	90,220	28,519,452	316	507	-	-	-
Unigene	Male	47,808	28,236,419	591	923	47,808	10,120	37,688
	Female	50,605	29,185,843	577	902	50,605	10,700	39,905
Merge	All	45,179	32,460,674	718	1116	45,179	12,483	32,696

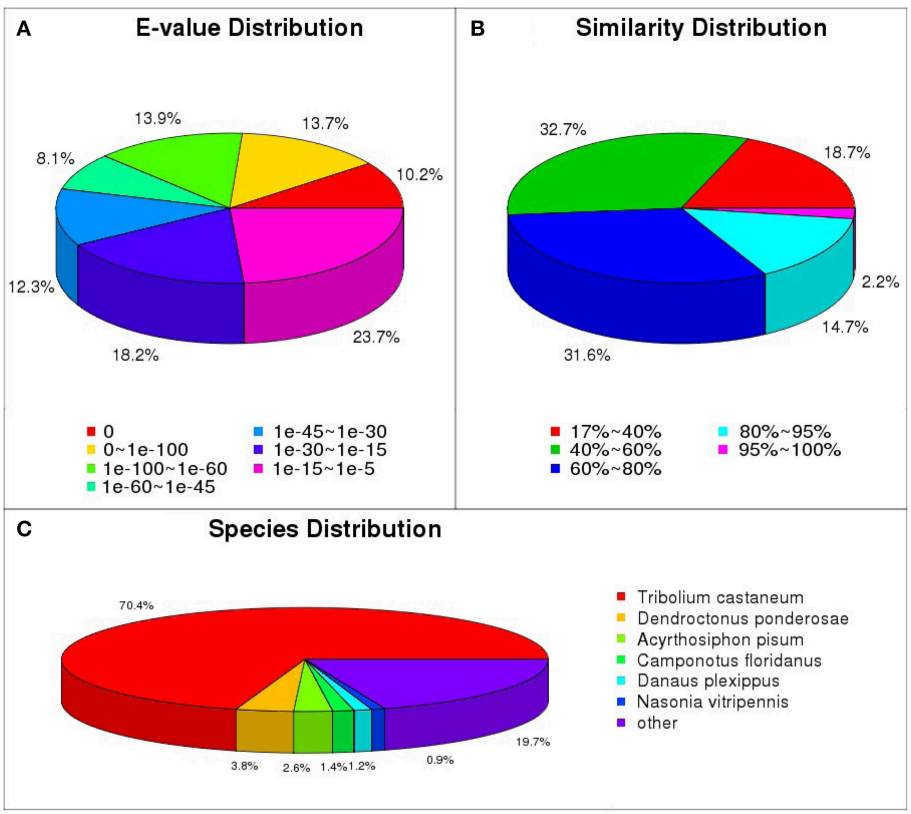

FIGURE 1 | Homology analyses of the *L. decemlineata* unigenes. All distinct gene sequences (24,880) that had blast annotations against the nr database with a cut-off *E*-value 10^{-5} were analyzed for **(A)** *E*-value distribution, **(B)** similarity distribution, and **(C)** species distribution.

Functional Annotation

The annotation of unigenes was performed by NCBI blastx against a pooled database of non-redundant (nr) and SwissProt protein sequences with an *e*-value cut-off of 1e-5 (Altschul et al., 1997). The blast results were then imported into the Blast2GO for GO Annotation (Conesa et al., 2005). Protein coding region prediction was performed by OrfPredictor (Min et al., 2005) according to the blast result. The signal peptide of the protein sequences were predicted using SignalP 4.0 (Petersen et al., 2011). The transmembrane-domains of annotated genes were predicted using TMHMM Server Version2.0 (http://www.cbs.dtu.dk/services/TMHMM) (Krogh et al., 2001).

Phylogenetic Analyses

The phylogenetic reconstruction implemented for the analysis of OR, IR, OBP, and CSP was performed based on the amino sequences of the candidate olfaction genes and the collected data sets. The OR data set contained OR sequences identified in Coleopteran species (239 from *T. castaneum* (Richards et al., 2008; Kim et al., 2010), 49 from *D. ponderosae* (Andersson et al., 2013), 42 from *I. typographus* (Andersson et al., 2013), and 57 from *M. caryae* (Mitchell et al., 2012). The IR data set contained 15, 7, and 66 IR sequences from *D. ponderosae* (Andersson et al., 2013), *I. typographus* (Andersson et al., 2013) and *D.melanogaster* (Croset et al., 2010), respectively. The OBP data set contained 46 sequences from *T. castaneum* (Richards et al., 2008; Kim et al., 2010), 31 sequences from *D. ponderosae* (Andersson et al., 2013), and 15 sequences from *I. typographus* (Andersson et al., 2013). The CSP data set contained the 40 sequences from *T. castaneum* (Richards et al., 2008; Kim et al., 2010), 11 sequences from *D. ponderosae* (Andersson et al., 2013), and 5 sequences from *I. typographus* (Andersson et al., 2013). The protein name and accession number of the genes used for phylogenetic tree building are listed in **Supplementary Material S1**. Amino acid sequences were aligned using MAFFT (https://www.ebi.ac.uk/Tools/msa/mafft/) (Katoh and Toh, 2008). Unrooted trees were constructed by the maximum-likelihood method in FastTree 2.1 software using the default parameters. To estimate reliability of each split in the tree, the local support values were computed based on the Shimodaira-Hasegawa (SH) test (Price et al., 2010) Dendrograms were created and colored in FigTree software (http://tree.bio.ed.ac.uk/software/figtree/).

Expression Analysis by Semi-quantitative Reverse Transcription PCR

To illustrate and compare the expression of candidate ORs in male and female antennae, semi-quantitative reverse transcription PCR (RT-PCR) was performed using cDNAs prepared from male and female antennae. *L. decemlineata* tissue samples were collected for three biological replicates. In each replicate, about two micrograms total RNA were extracted from approximately 100 antennae of male or female adults as mentioned above. Prior to cDNA synthesis, RNA were treated with DNase I to remove trace amounts of genomic DNA. The cDNA was synthesized by First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) and was used as a template in PCR reactions with gene-specific primers. *Ribosomal protein L31* (*LdecRL31*) and *ribosomal protein S3* (*LdecRPS3*) were used as controls. Primers were designed using the Primer Premier 5 software (PREMIER Biosoft International). The primer

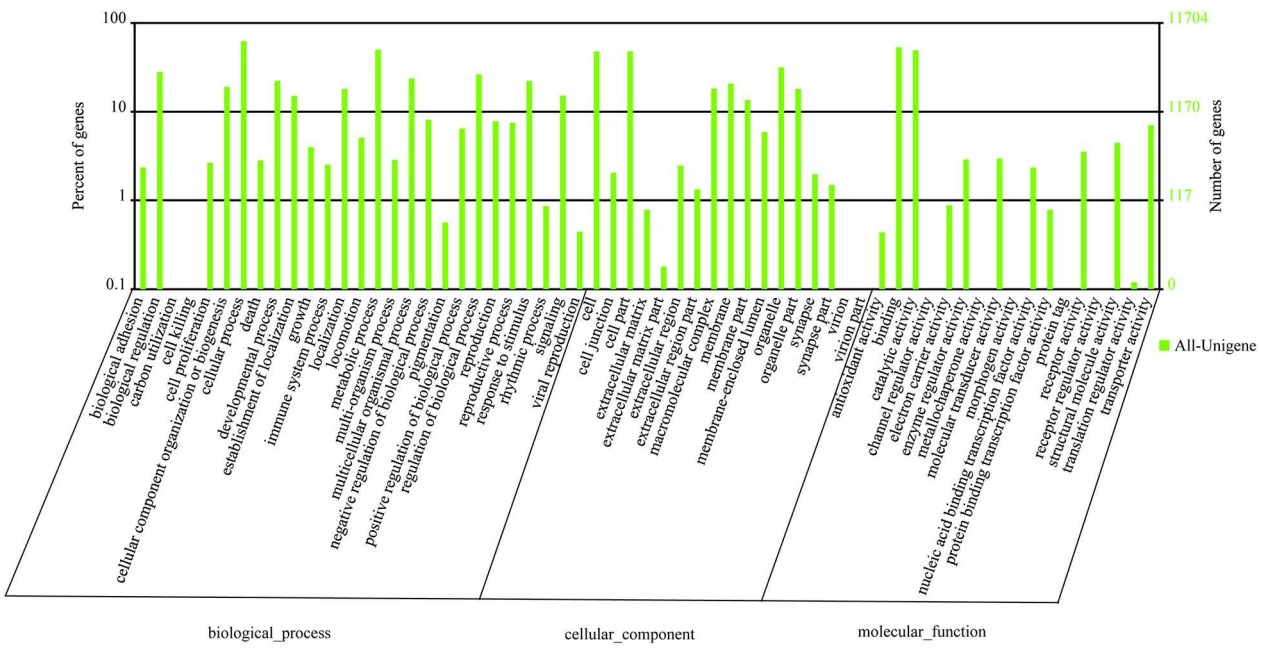

FIGURE 2 | Gene ontology distributions of *L. decemlineata* unigenes annotated at GO level 2. The Y-axis shows the percentage and number of annotated GO terms in three categories: biological process, cellular component, and molecular function. The X-axis shows three areas of annotation, and in each area the sequences are further divided into subgroups at GO level 2.

TABLE 2 | Unigenes of candidate odorant binding proteins.

Unigene reference	Gene name	Length (bp)	ORF (aa)	Blastx best hit (Reference/Name/Species)	*E*-value	Identity	Status	Signal Peptide
Unigene20025	LdecOBP1	786	255	gb|AGI05158.1|odorant-binding protein 2 [Dendroctonus ponderosae]	1.00E-56	0.37	Complete ORF	Yes
Unigene19829	LdecOBP2	900	248	gb|AGI05159.1|odorant-binding protein 21 [Dendroctonus ponderosae]	8.00E-53	0.4	Complete ORF	Yes
CL1269.Contig1	LdecOBP3	635	176	gb|AFI45057.1|odorant-binding protein [Dendroctonus ponderosae]	3.00E-08	0.27	5′ missing	No
Unigene3701	LdecOBP4	609	176	gb|AHB59657.1|odorant-binding protein 4 [Sogatella furcifera]	1.00E-50	0.52	3′ missing	Yes
CL3396.Contig2	LdecOBP5	523	159	gb|EFA02857.1|odorant binding protein 12 [Tribolium castaneum]	6.00E-25	0.33	Complete ORF	Yes
Unigene1581	LdecOBP6	617	149	gb|AFI45057.1|odorant-binding protein [Dendroctonus ponderosae]	8.00E-07	0.24	Complete ORF	Yes
Unigene22758	LdecOBP7	577	144	gb|AGO28153.1|odorant binding protein 2 [Bactrocera dorsalis]	7.00E-10	0.29	Complete ORF	Yes
Unigene17711	LdecOBP8	782	143	gb|ADD70031.1|minus-C odorant binding protein 2 [Batocera horsfieldi]	1.00E-20	0.35	Complete ORF	Yes
Unigene18256	LdecOBP9	593	143	gb|AHA33380.1|odorant-binding protein 2 [Batocera horsfieldi]	6.00E-55	0.6	Complete ORF	Yes
Unigene19973	LdecOBP10	645	143	gb|AHA33382.1odorant-binding protein 1 [Batocera horsfieldi]	1.00E-61	0.63	Complete ORF	Yes
CL373.Contig2	LdecOBP11	571	142	gb|AGM38609.1|odorant binding protein [Chilo suppressalis]	2.00E-08	0.28	Complete ORF	Yes
Unigene15355	LdecOBP12	693	139	gb|EFA10803.1|odorant binding protein 23 [Tribolium castaneum]	2.00E-45	0.56	Complete ORF	Yes
CL1566.Contig1	LdecOBP13	673	136	gb|EFA04594.1|odorant binding protein 6 [Tribolium castaneum]	3.00E-55	0.72	Complete ORF	Yes
Unigene13766	LdecOBP14	491	135	gb|EFA07546.1|odorant binding protein C03, partial [Tribolium castaneum]	4.00E-28	0.43	Complete ORF	Yes
Unigene18285	LdecOBP15	651	134	gb|ADD82417.1|minus-C odorant binding protein 4 [Batocera horsfieldi]	7.00E-35	0.44	Complete ORF	Yes
Unigene18159	LdecOBP16	402	133	ref|XP_008200270.1|PREDICTED: general odorant-binding protein 28a [Tribolium castaneum]	6.00E-20	0.4	5′,3′ missing	Yes
Unigene4434	LdecOBP17	524	133	gb|EFA07430.1|odorant binding protein (subfamily minus-C) C04 [Tribolium castaneum]	2.00E-17	0.34	Complete ORF	Yes
Unigene6224	LdecOBP18	636	132	gb|EFA02826.1|odorant binding protein C15 [Tribolium castaneum]	5.00E-16	0.37	Complete ORF	Yes
Unigene11398	LdecOBP19	609	132	gb|AGI05182.1|odorant-binding protein 29 [Dendroctonus ponderosae]	9.00E-15	0.41	Complete ORF	Yes
CL2715.Contig1	LdecOBP20	1143	131	gb|EFA04594.1|odorant binding protein 6 [Tribolium castaneum]	4.00E-40	0.5	Complete ORF	Yes
Unigene13119	LdecOBP21	798	130	gb|EFA07544.1|odorant binding protein (subfamily minus-C) C01 [Tribolium castaneum]	6.00E-18	0.35	Complete ORF	Yes
Unigene16073	LdecOBP22	464	128	gb|EFA05742.1|odorant binding protein 4 [Tribolium castaneum]	5.00E-12	0.33	Complete ORF	Yes
Unigene18306	LdecOBP23	427	125	gb|EFA05742.1|odorant binding protein 4 [Tribolium castaneum]	1.00E-06	0.29	Complete ORF	Yes
Unigene20476	LdecOBP24	510	122	gb|AGI05186.1|odorant-binding protein 16 [Dendroctonus ponderosae]	2.00E-13	0.31	Complete ORF	Yes
Unigene13748	LdecOBP25	413	120	gb|ADD82417.1|minus-C odorant binding protein 4 [Batocera horsfieldi]	5.00E-09	0.31	Complete ORF	Yes
Unigene30854	LdecOBP26	312	67	gb|EFA05695.1|odorant binding protein 11 [Tribolium castaneum]	2.00E-26	0.71	5′ missing	No

sequences are available in **Supplementary Material S2**. Taq MasterMix (CWBIO, Beijing, China) was used for PCR reactions under general 3-step amplification of 94°C for 30 s, 53°C for 30 s, 72°C for 30 s. The PCR cycle-numbers were adjusted respectively for each gene. For OR, cycle-numbers ranged from 38 to 40. For high-express-level control genes *LdecRL31* and *LdecRPS3*, cycle-numbers were reduced to 28. PCR products were run on a 2% agarose gel and verified by DNA sequencing. In the negative control, the cDNA template was replaced by water.

Results

Sequencing and Unigene Assembly

Using an Illumina HiSeq 2000 90PE RNA-Seq strategy, a total of 56.75 million and 59.19 million raw-reads were obtained respectively from the libraries of male and female antenna. After removing low quality and adaptor reads, 51.43 million and 52.36 million clean-reads were generated. The total bases of sequence data were approximately 4.63 and 4.71 gigabases from male and female samples, respectively. The clean reads of the *L. decemlineata* antennal transcriptome were deposited in the NCBI SRA database, under the accession number of SRX974484 (male) and SRX974488 (female). The clean-reads were assembled into 47,808 and 50,605 unigenes separately for male and female. All unigenes were merged and clustered into the final 45,179 unigenes consisting of 12,483 distinct clusters and 32, 696 distinct singletons. The transcript dataset was 32.46 megabases in size and with a mean length of 718 nt and N50 of 1, 116 nt. 10,756 unigenes were larger than 1000 nt in length, which comprised 23.81% of all unigenes (**Table 1**).

Gene Identification and Functional Annotation

The functional annotations of the unigenes were performed mainly based on the blastx results against the nr database. Through annotation by blastx, 24,880 (55.1%) unigenes matched to known proteins. Among the 24,880 annotated

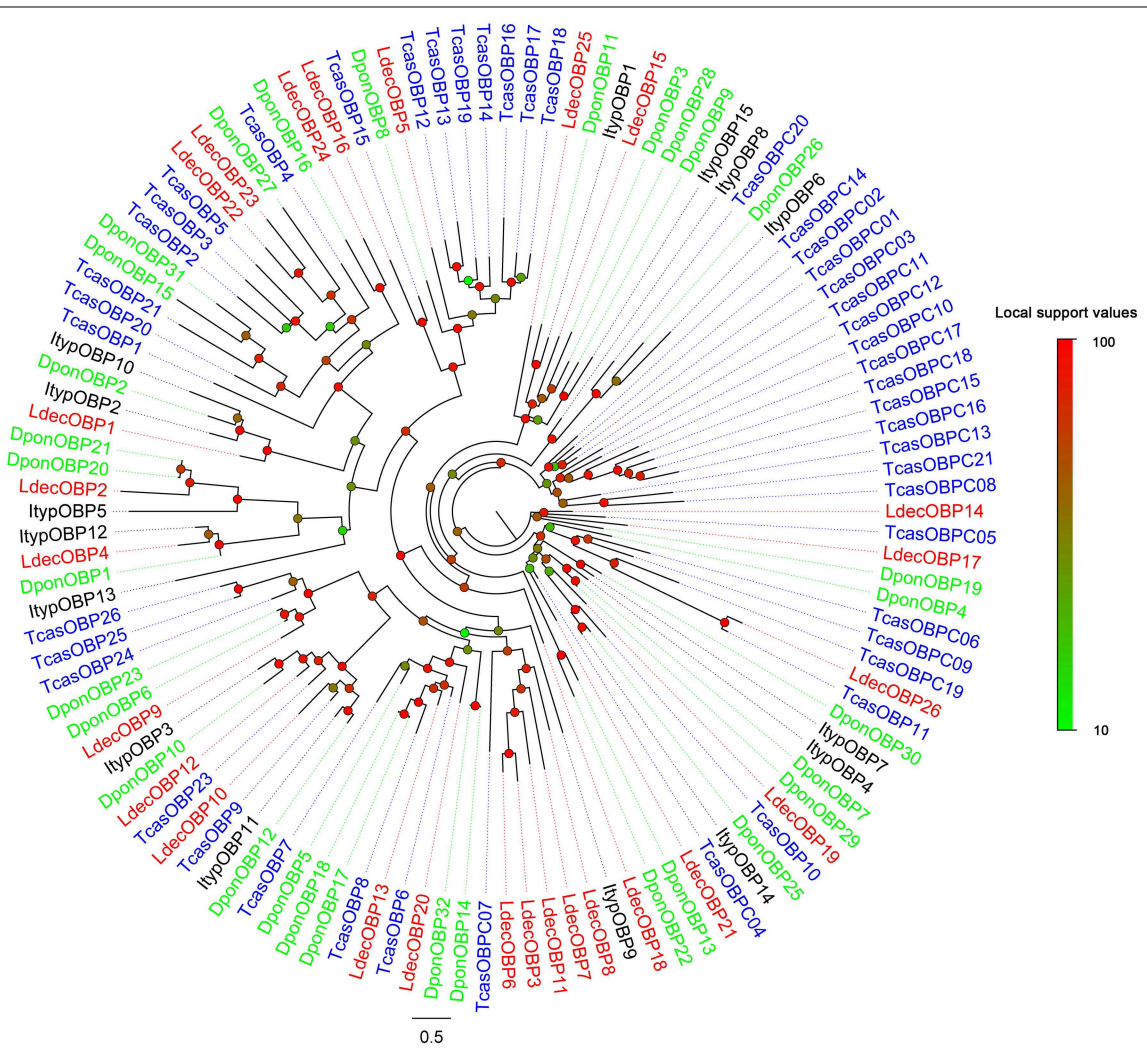

FIGURE 3 | Phylogenetic tree of candidate LdecOBPs with known Coleopteran OBP sequences. Tcas, *T. castaneum*; Dpon, *D. ponderosae*; Ityp, *I. typographus*.

unigenes, 14,463 (58.1%) showed strong homology (*e*-value smaller than 1e-45), whereas 5897 (23.7%) showed poor matches with *e*-value between 1e-15 and 1e-5 (**Figure 1A**). The similarity comparison showed 12,089 (48.6%) unigenes have more than 60% similarity with known proteins (**Figure 1B**). Blast analysis showed that 70.4% of the annotated unigenes matched with *T. castaneum*, followed by *D. ponderosae* (3.8%), *Acyrthosiphon pisum* (2.6%) and *Camponotus floridanus* (1.4%) (**Figure 1C**).

Gene ontology (GO) annotation of the unigene set was obtained using Blast2GO pipeline according to the blastx search against nr. From the 45,179 final unigenes set, a total of 11,704 unigenes were assigned various GO terms. In the molecular function category, the genes expressed in the antennae were mostly enriched to binding activity (e.g., nucleotide, ion, and odorant binding) and catalytic activity (e.g., hydrolase and oxidoreductase). In the biological process terms, cellular, and metabolic processes were the most represented. In the cellular component terms, cell, cell part, and organelle were the most abundant (**Figure 2**).

Identification of Putative Odorant Binding Proteins

Within the *L. decemlineata* antennal transcriptome, 26 different sequences encoding odorant binding proteins were identified. Sequence analysis identified all but four transcripts (LdecOBP3, LdecOBP4, LdecOBP16, and LdecOBP26) with a full length ORF. The signal peptide, which is a typical structure of OBPs was not found in only two LdecOBPs (LdecOBP3 and LdecOBP26), due to incomplete N-termini. The length of all full-length LdecOBPs ranged from 122 to 255 amino acids. Compared to the ORs, insect OBPs are more highly conserved. The similarity between the LdecOBPs and known OBP of other insects was relatively low. Only seven predicted OBPs (LdecOBP4, LdecOBP9, LdecOBP10, LdecOBP12, LdecOBP13, LdecOBP20, and LdecOBP26) have more than 50% similarity with OBPs from *T. castaneum* or *Batocera horsfieldi* (**Table 2**). In our phylogenetic analysis of the OBPs in different beetles, LdecOBPs are spread across various branches (**Figure 3**) where they generally formed small subgroups together with OBPs from other three beetles. These splits were strongly

TABLE 3 | Unigenes of candidate chemosensory proteins.

Unigene reference	Gene name	Length (bp)	ORF (aa)	Blastx best hit (Reference/Name/Species)	*E*-value	Identity	Status	Signal Peptide
CL3420.Contig2	LdecCSP1	778	195	ref|NP_001039288.1|chemosensory protein 6 precursor [Tribolium castaneum]	3.00E-37	0.38	Complete ORF	No
Unigene20159	LdecCSP2	1511	149	ref|NP_001039287.1|chemosensory protein 5 precursor [Tribolium castaneum]	5.00E-28	0.44	Complete ORF	Yes
Unigene18988	LdecCSP3	519	131	ref|NP_001039279.1|chemosensory protein 11 precursor [Tribolium castaneum]	9.00E-37	0.47	Complete ORF	Yes
Unigene20037	LdecCSP4	787	131	gb|AEC04842.1|chemosensory protein [Batocera horsfieldi]	4.00E-53	0.62	Complete ORF	Yes
Unigene8858	LdecCSP5	576	127	ref|NP_001039276.1|chemosensory protein 19 precursor [Tribolium castaneum]	2.00E-44	0.66	Complete ORF	Yes
Unigene11342	LdecCSP6	534	127	ref|NP_001039280.1|chemosensory protein 12 precursor [Tribolium castaneum]	3.00E-49	0.57	Complete ORF	Yes
CL1466.Contig3	LdecCSP7	837	125	ref|NP_001039279.1|chemosensory protein 11 precursor [Tribolium castaneum]	3.00E-35	0.56	Complete ORF	Yes
Unigene11467	LdecCSP8	411	124	ref|NP_001039289.1|chemosensory protein 7 precursor [Tribolium castaneum]	1.00E-55	0.67	3′ missing	Yes
Unigene4499	LdecCSP9	546	123	gb|AEC04843.1|chemosensory protein [Batocera horsfieldi]	1.00E-59	0.72	Complete ORF	Yes
Unigene15973	LdecCSP10	632	119	gb|AGI05164.1|chemosensory protein 8 [Dendroctonus ponderosae]	1.00E-41	0.53	Complete ORF	Yes
Unigene13099	LdecCSP11	680	115	ref|XP_008200934.1|PREDICTED: chemosensory protein 1 isoform X1 [Tribolium castaneum]	6.00E-43	0.67	Complete ORF	Yes
Unigene22587	LdecCSP12	368	113	gb|AGI05172.1|chemosensory protein 2 [Dendroctonus ponderosae]	8.00E-38	0.5	3′ missing	Yes
Unigene23091	LdecCSP13	241	76	ref|XP_008193776.1|PREDICTED: chemosensory protein 6 isoform X2 [Tribolium castaneum]	3.00E-20	0.58	3′ missing	No
Unigene5339	LdecCSP14	290	69	ref|NP_001039284.1|chemosensory protein 17 precursor [Tribolium castaneum]	3.00E-13	0.41	3′ missing	No
Unigene32053	LdecCSP15	210	69	ref|NP_001039290.1|chemosensory protein 8 precursor [Tribolium castaneum]	2.00E-20	0.63	5′,3′ missing	Yes

supported by high local support values. A species specific branch consisting of 5 OBPs from *L. decemlineata* (LdecOBP3, LdecOBP6, LdecOBP7, LdecOBP8, and LdecOBP11) that is divergent from OBPs of other insects has been identified; these specific LdecOBPs might have some key species specific function.

The information, including unigene reference, length, and best blastx hit of all the 26 LdecOBPs are listed in **Table 2**. The sequences of all 26 LdecOBPs are listed in **Supplementary Material S3**.

Identification of Putative Chemosensory Proteins

Bioinformatic analysis led to the identification 15 different sequences encoding candidate CSPs in the *L. decemlineata* antennal transcriptome. Sequence analysis identified ten unigenes with a full length ORF with a predicted signal peptide sequence (**Table 3**).

Compared to OBPs, the conservation of CSPs of different Coleopteran was relatively high. Two thirds (10) of the LdecCSPs had more than 50% similarities with other CSPs (**Table 3**). The phylogenetic analyses also indicated

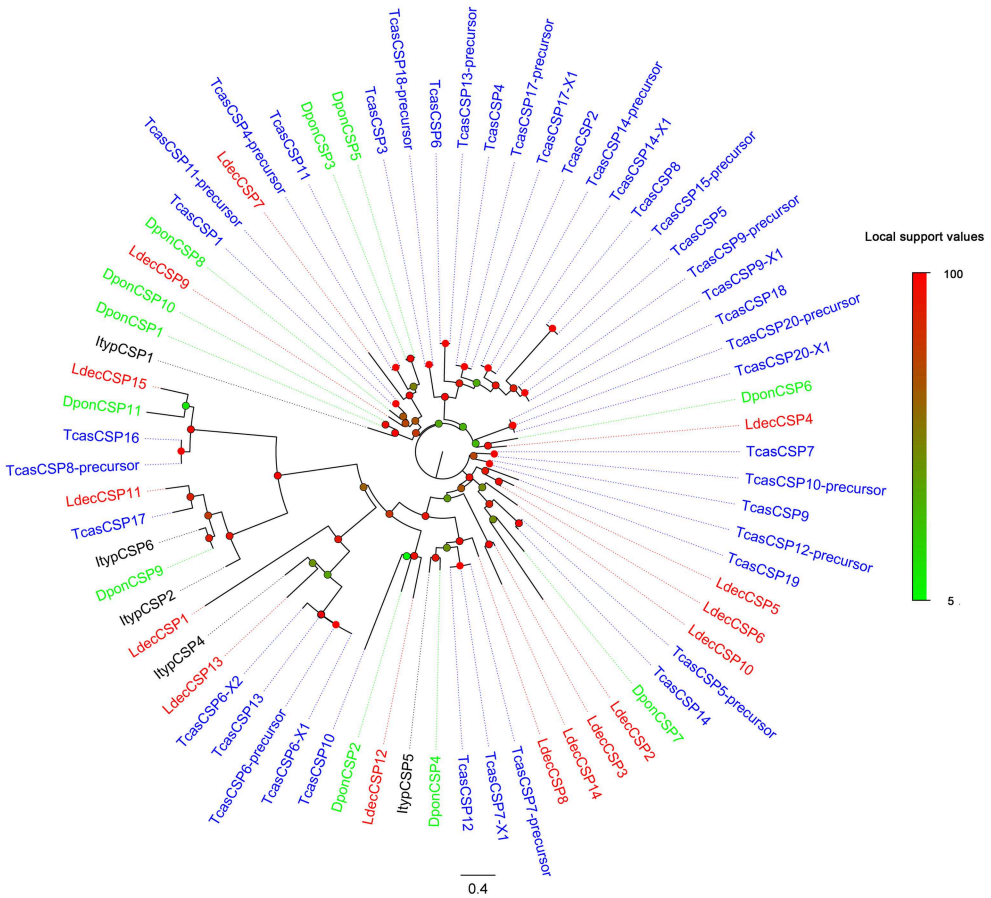

FIGURE 4 | Phylogenetic tree of candidate LdecCSPs with known Coleopteran CSP sequences. Tcas, *T. castaneum*; Dpon, *D. ponderosae*; Ityp, *I. typographus*.

TABLE 4 | Unigenes of candidate sensory neuron membrane proteins.

Unigene reference	Gene name	Length (bp)	ORF (aa)	Blastx best hit (Reference/Name/Species)	*E*-value	Identity	Status
Unigene1678	LdecSNMP1	1856	531	gb|AFI45066.1|sensory neuron membrane protein [Dendroctonus ponderosae]	0	0.51	Complete ORF
Unigene17817	LdecSNMP2	2244	526	ref|XP_001816436.1|PREDICTED: sensory neuron membrane protein 1 [Tribolium castaneum]	0	0.59	Complete ORF
Unigene1763	LdecSNMP3	1189	395	ref|XP_970008.1|PREDICTED: sensory neuron membrane protein 2 [Tribolium castaneum]	1.00E-91	0.4	5′,3′ missing

conservation of Coleopteran CSPs (**Figure 4**). Most candidate LdecCSPs clustered with orthologs of *T. castaneum*, *D. ponderosae* and *I. typographus* into a separate clade. Only 2 LdecCSPs (LdecCSP2 and LdecCSP3) formed one small subgroup. Only one sequence-LdecCSP15 had low local support value unable to clearly demonstrate their phylogenetic positions.

The information, including unigene reference, length, and best blastx hit of all the LdecCSPs are listed in **Table 3**. The sequences of all 15 LdecCSPs are listed in **Supplementary Material S3**.

Identification of Candidate Sensory Neuron Membrane Proteins

We found three SNMPs (LdecSNMP1-3) in our transcriptome. Two of them were predicted to have full-length ORF. Both LdecSNMP1 and LdecSNMP2 had more than 50% (51 and 59%) identity with SNMP of *D. ponderosae* and *T. castaneum*. LdecSNMP3 had only 40% similarity with SNMP2 of *T. castaneum* (**Table 4**).

The information, including unigene reference, length, and best blastx hit of all the three SNMPs are listed in **Table 4**. The sequences of all three SNMPs were listed in **Supplementary Material S3**.

Identification of Candidate Odorant Receptors

The unigenes related to candidate OR were identified by keyword search of the blastx annotation. We identified 37 distinct unigenes that were putative OR genes. Of these, a full-length *LdecOrco* gene coding 479 amino acids was easily identified because it had intact open reading frames and seven transmembrane domains, which are characteristic of typical insect ORs. The 36 predicted incomplete ORs were of short length and only three of them contained a deduced protein longer than 300 amino acids. The deduced protein length of 24 ORs were even shorter than 200 amino acids.

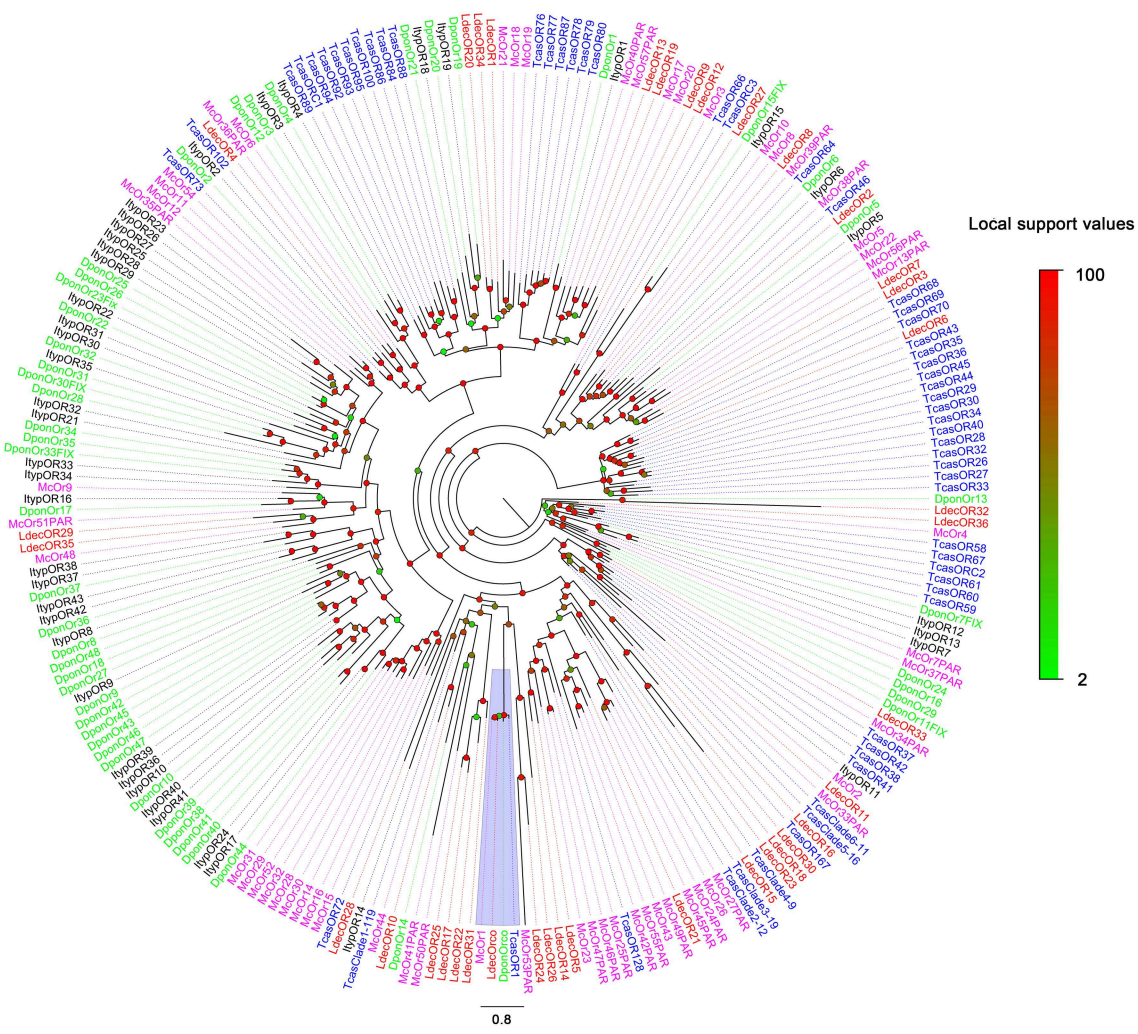

FIGURE 5 | Phylogenetic tree of candidate LdecORs with known Coleopteran OR sequences. Tcas, *T. castaneum*; Dpon, *D. ponderosae*; Ityp, *I. typographus*; Ma, *M. caryae.* The clade in purple indicates the co-receptor gene clade.

TABLE 5 | Unigenes of candidate odorant receptors.

Unigene reference	Gene name	Length (bp)	ORF (aa)	Blastx best hit (Reference/Name/Species)	*E*-value	Identity	Status	TMD (No)
CL543.Contig1	LdecOrco	5613	479	gb|EFA05687.1|odorant receptor 1 [Tribolium castaneum]	0	0.86	Complete ORF	7
CL3611.Contig2	LdecOR1	1180	346	gb|EFA10702.1|odorant receptor 89 [Tribolium castaneum]	7.00E-54	0.29	5′ missing	6
CL1619.Contig2	LdecOR2	1171	317	gb|EFA10800.1|odorant receptor 64 [Tribolium castaneum]	8.00E-25	0.37	Complete ORF	6
CL1005.Contig3	LdecOR3	1127	302	gb|EFA10800.1|odorant receptor 64 [Tribolium castaneum]	1.00E-29	0.3	5′ missing	4
CL5234.Contig2	LdecOR4	910	289	ref|XP_966790.1|PREDICTED: odorant receptor 82a [Tribolium castaneum]	3.00E-84	0.48	5′ missing	4
Unigene19868	LdecOR5	852	244	gb|EEZ99418.1|odorant receptor 119 [Tribolium castaneum]	5.00E-18	0.28	5′ missing	2
Unigene12292	LdecOR6	782	240	gb|EEZ99411.1|odorant receptor 43 [Tribolium castaneum]	2.00E-83	0.52	5′ missing	3
Unigene9	LdecOR7	1596	237	gb|EFA10800.1|odorant receptor 64 [Tribolium castaneum]	6.00E-34	0.35	5′ missing	4
Unigene21742	LdecOR8	696	232	gb|EFA10800.1|odorant receptor 64 [Tribolium castaneum]	2.00E-91	0.6	5′,3′ missing	4
Unigene6386	LdecOR9	960	216	gb|EFA10778.1|odorant receptor 78 [Tribolium castaneum]	1.00E-36	0.29	5′ missing	3
Unigene24756	LdecOR10	640	213	ref|XP_008200336.1|PREDICTED: odorant receptor 82a-like isoform X2 [Tribolium castaneum]	5.00E-34	0.66	5′,3′ missing	3
Unigene15568	LdecOR11	591	198	ref|XP_008198156.1|PREDICTED: putative odorant receptor 71a [Tribolium castaneum]	1.00E-07	0.24	5′,3′ missing	3
Unigene17958	LdecOR12	623	194	gb|EFA10779.1|odorant receptor 76 [Tribolium castaneum]	2.00E-36	0.38	5′ missing	0
Unigene2150	LdecOR13	693	190	gb|EFA10779.1|odorant receptor 76 [Tribolium castaneum]	1.00E-26	0.33	5′ missing	2
Unigene6229	LdecOR14	699	184	gb|AGS43053.1|odorant receptor Or2d [Cephus cinctus]	3.00E-18	0.29	5′ missing	2
CL2511.Contig2	LdecOR15	1010	182	gb|EFA05789.1|odorant receptor 113 [Tribolium castaneum]	2.00E-16	0.26	5′ missing ′	0
Unigene18201	LdecOR16	540	180	ref|XP_001812261.1|PREDICTED: odorant receptor 49b-like [Tribolium castaneum]	5.00E-31	0.37	5′,3′ missing	3
Unigene5208	LdecOR17	535	178	gb|EFA02941.1|odorant receptor 93 [Tribolium castaneum]	6.00E-21	0.31	5′,3′ missing	2
CL3976.Contig3	LdecOR18	506	168	gb|EFA07574.1|odorant receptor 151 [Tribolium castaneum]	3.00E-08	0.27	5′,3′ missing	3
CL3467.Contig2	LdecOR19	671	168	gb|EFA10801.1|odorant receptor 94 [Tribolium castaneum]	1.00E-21	0.32	5′ missing	1
Unigene9109	LdecOR20	787	158	gb|EFA10702.1|odorant receptor 89 [Tribolium castaneum]	1.00E-26	0.34	5′ missing	0
Unigene19131	LdecOR21	506	154	ref|XP_006558397.1|PREDICTED: putative odorant receptor 13a-like, partial [Apis mellifera]	3.00E-15	0.3	5′ missing	2
Unigene14111	LdecOR22	460	153	gb|EFA02873.1|odorant receptor 92 [Tribolium castaneum]	1.00E-18	0.33	3′ missing	3
Unigene13563	LdecOR23	455	151	gb|EFA05790.1|odorant receptor 114 [Tribolium castaneum]	3.00E-07	0.28	5′,3′ missing	3
Unigene30834	LdecOR24	443	147	emb|CAM84002.1|olfactory receptor 4 [Tribolium castaneum]	1.00E-19	0.35	5′,3′ missing	2
Unigene9968	LdecOR25	405	135	ref|XP_008197941.1|PREDICTED: odorant receptor 67c-like [Tribolium castaneum]	2.00E-13	0.34	5′,3′ missing	1
Unigene19594	LdecOR26	388	129	gb|EEZ99311.1|odorant receptor 69 [Tribolium castaneum]	6.00E-18	0.35	5′,3′ missing	1

(Continued)

TABLE 5 | Continued

Unigene reference	Gene name	Length (bp)	ORF (aa)	Blastx best hit (Reference/Name/Species)	*E*-value	Identity	Status	TMD (No)
Unigene20484	LdecOR27	450	125	gb|ABK27853.1|odorant receptor 45 [Bombyx mori]	1.00E-12	0.31	5′ missing	3
Unigene17010	LdecOR28	369	123	gb|AFC91733.1|putative odorant receptor OR25 [Cydia pomonella]	3.00E-06	0.26	5′,3′ missing	2
Unigene14947	LdecOR29	481	123	gb|AGI05173.1|odorant receptor 23 [Dendroctonus ponderosae]	1.00E-12	0.35	5′ missing	1
Unigene10946	LdecOR30	369	122	gb|EFA02801.1|odorant receptor 167 [Tribolium castaneum]	4.00E-13	0.29	5′,3′ missing	1
Unigene2944	LdecOR31	418	121	gb|EFA10801.1|odorant receptor 94 [Tribolium castaneum]	8.00E-08	0.29	3′ missing	2
Unigene10594	LdecOR32	356	118	ref|XP_001814862.1|PREDICTED: odorant receptor 82a [Tribolium castaneum]	5.00E-09	0.3	5′,3′ missing	2
Unigene3179	LdecOR33	340	112	gb|EEZ99229.1|odorant receptor 37 [Tribolium castaneum]	1.00E-39	0.62	5′,3′ missing	2
Unigene16792	LdecOR34	480	104	gb|EFA10702.1|odorant receptor 89 [Tribolium castaneum]	1.00E-22	0.42	5′ missing	2
Unigene21476	LdecOR35	377	103	gb|AGI05173.1|odorant receptor 23 [Dendroctonus ponderosae]	5.00E-16	0.4	5′ missing	1
CL3422.Contig1	LdecOR36	464	100	gb|EEZ99171.1|odorant receptor 59 [Tribolium castaneum]	1.00E-29	0.55	5′,3′ missing	1

The blastx results showed that the identities of these predicted ORs with known insect ORs is quite low. Only six predicted ORs (LdecOrco, LdecOR6, LdecOR8, LdecOR10, LdecOR33, and LdecOR36) have greater than 50% identity with ORs from *T. castaneum*. Even the LdecOrco had only 86% identity with the Orco from *T. castaneum*. Phylogenetic analysis was performed with ORs from *T. castaneum*, *D. ponderosae*, *I. typographus* and *M. caryae*. The results once again suggest high divergence of the OR genes (**Figure 5**). The branch of Orco was easily detected as it has a high degree of identity. All of the other LdecORs were distributed in different branches of the phylogenetic tree. A species-specific branch was identified consisting of four ORs from *L. decemlineata* (LdecOR17, LdecOR22, LdecOR25, and LdecOR31) that was clearly divergent from other ORs. Four LdecORs (LdecOR16, LdecOR18, LdecOR23, and LdecOR30) showed close relation to OR167 from *T. castaneum*, and these five ORs formed a distinct subgroup. Most of the splits in the tree were supported by high local support values and only a few splits were not reliable.

Information, including unigene reference, length, and best blastx hit of all 37 OR are listed in **Table 5**. The sequences are listed in **Supplementary Material S3**.

Identification of Candidate Ionotropic Receptors

The putative IR genes in the *L. decemlineata* antennal transcriptome were represented according to their similarity to known insect IRs. Bioinformatic analysis led to the identification of ten candidate IRs, all ten sequences are marked as incomplete due to lacking a complete 5′ or 3′ terminus. The insect IRs contained three transmembrane domains (Benton et al., 2009). TMHMM2.0 predicted nine candidate IRs with different numbers of transmembrane domains (**Table 6**). One candidate IR was deemed to be an IR8a homolog due to its high identity (59%) to DponIR8a. A candidate IR25a homolog was also easily identified. The subgroup of IR75q2 is likely to extend to *L. decemlineata*, as four transcripts had high identity to IR75q2 homologs from *C. pomonella*, *S. littoralis,* and *Aedes aegypti*. Two IR76b homologs (LdecIR76b.1 and LdecIR76b.2) were also detected. The remaining two LdecIRs have similarity with IR87a and IR93a from *D. melanogaster*, respectively. In the phylogenetic tree of IRs, all *L. decemlineata* IR candidates clustered with their ionotropic receptor orthologs into separate sub-clades (**Figure 6**). Because of the relative high conservation of IRs, all the splits of LdecIRs were strongly supported by high local support values. The information, including unigene reference, length, and best blastx hit of all the ten IRs are listed in **Table 6**. The sequences of all 20 IRs were listed in **Supplementary Material S3**.

Sex-specific Expression of Candidate *L. Decemlineata* or Genes

The expression patterns of the candidate 37 ORs in male and female antennae were analyzed by RT-PCR. Results for all of these genes are listed in **Figure 7**. The RT-PCR results showed all of the 37 *LdecORs* expressed in the antennae, but the expression level was quite low. For the control genes *LdecRL31* and *LdecRPS3*, the 28 cycle of amplification was sufficient for detection. Conversely, for all the candidate *LdecORs* (including *LdecOrco*), the bands were difficult to detect unless the cycle-numbers increased to 38. One candidate OR- *LdecOR6* was detected to expressed only in male antennae. Except *LdecOR6*, the expressions of all the other candidate ORs were detected in both male and female antennae. The expression of *LdecOR5*, *LdecOR12*, *LdecOR26,* and *LdecOR32* was clearly higher in male compared to female, and *LdecOR3* and *LdecOR29* expressed higher in female.

TABLE 6 | Unigenes of candidate ionotropic receptors.

Unigene reference	Gene name	Length (bp)	ORF (aa)	Blastx best hit (Reference/Name/Species)	*E*-value	Identity	Status	TMD (No)
CL3955.Contig1	LdecIR8a	2689	875	gb|AGI05169.1|ionotropic receptor 8a [Dendroctonus ponderosae]	0	0.59	5′ missing	3
Unigene15982	LdecIR25a	2098	699	gb|AFC91757.1|putative ionotropic receptor IR25a [Cydia pomonella]	0	0.68	5′,3′ missing	2
Unigene22363	LdecIR87a	2051	630	gb|AFC91760.1|putative ionotropic glutamate receptor 87a, partial [Cydia pomonella]	4.00E-23	0.22	5′ missing	6
CL2971.Contig3	LdecIR75q.2.1	2007	554	gb|ADR64685.1|putative chemosensory ionotropic receptor IR75q.2 [Spodoptera littoralis]	8.00E-57	0.41	5′ missing	2
Unigene2581	LdecIR75q.2.2	830	276	ref|XP_001648018.1|ionotropic glutamate receptor invertebrate [Aedes aegypti]	6.00E-44	0.42	5′,3′ missing	1
Unigene12027	LdecIR76b.1	814	271	gb|ETN63667.1|Ionotropic receptor 76b [Anopheles darlingi]	1.00E-75	0.51	5′,3′ missing	3
Unigene5182	LdecIR76b.2	739	246	gb|AFC91765.1|putative ionotropic receptor IR76b [Cydia pomonella]	1.00E-38	0.39	5′,3′ missing	1
Unigene9077	LdecIR93a	962	160	gb|AGY49252.1|putative ionotropic receptor, partial [Sesamia inferens]	1.00E-41	0.56	5′ missing	0
Unigene5590	LdecIR75q.2.3	447	149	gb|AFC91752.1|putative ionotropic receptor IR75q2 [Cydia pomonella]	7.00E-48	0.62	5′,3′ missing	1
Unigene782	LdecIR75q.2.4	364	121	gb|AFC91752.1|putative ionotropic receptor IR75q2 [Cydia pomonella]	3.00E-31	0.47	5′,3′ missing	1

Discussion

In this study, we annotated olfactory genes in a Coleopteran pest, *L. decemlineata*, through antennal transcriptome sequence. Compared with six previously reported beetle antennal transcriptomes (Mitchell et al., 2012; Andersson et al., 2013; Wang et al., 2014; Diakite et al., 2015) sequenced by 454 or Illumina platform, the depth of sequencing of this *L. decemlineata* antennal transcriptome was greater. The length of the assembled transcripts varied obviously in these seven beetles and the N50 of our transcripts is longer than those in *M. caryae* (Mitchell et al., 2012), *I. typographus* (Andersson et al., 2013), *M. alternatus* (Wang et al., 2014) and *D. helophoroides* (Wang et al., 2014), but shorter than the transcripts in *D. ponderosae* (Andersson et al., 2013). The high quality of our transcriptome sequencing laid the foundation for olfactory gene annotation.

The functional annotation of all the unigenes was first perform by different methods. The blastx results showed that 70.4% of the annotated unigenes matched with *T. castaneum*, whose genome is available and a large number of genes including olfactory genens have been identified and annotated. Compared with *T. castaneum*, there are relatively fewer genes of other Coleopteran published in Genbank. Compared with previous antennal transcriptomes in *I. typographus*, *D. ponderosae* (Andersson et al., 2013), *H. armigera* (Liu et al., 2012) and *C. suppressalis* (Cao et al., 2014), the enriched GO terms in each of the three categories were almost exactly the same as those observed in Coleopteran and Lepidopteran.

Within the *L. decemlineata* antennal transcriptome, a total of 26 OBP genes were predicted. In *T. castaneum*, there were a total of 46 OBPs identified through genome annotation (Richards et al., 2008; Kim et al., 2010). Previous studies have shown that some OBPs express specifically in non-antenna tissues (Gong et al., 2009), so the number OBPs annotated by antennal transcriptome sequence might be much less. Transcriptome analysis of *D. ponderosae* found a total of 31 candidate OBPs, but one third of them were not detected in the antennal cDNA library (Andersson et al., 2013). And in *I. typographus*, *M. alternatus*, *D. helophoroides,* and *R. dominica*, 15, 29, 23, and 16 transcripts encoding putative OBPs were annotated (Andersson et al., 2013; Wang et al., 2014; Diakite et al., 2015). Therefore, the number of LdecOBPs identified in this study is consistent with previous reports. The length of all full-length LdecOBPs (122–255 amino acids) is also in a reasonable range compared to OBPs of other insects (Hekmat-Scafe et al., 2002; Zhou et al., 2008; Gong et al., 2009; Liu et al., 2012; Cao et al., 2014) (**Table 2**).

The CSPs are another class of soluble proteins in the sensillum lymph with abundant expression (Foret et al., 2007). 15 CSP genes were identified in this study. There are a total of 40 CSPs including 15 precursors that were annotated from *T. castaneum* genome (Richards et al., 2008; Kim et al., 2010). And 11 (four transcripts were not found in the antenna), 6, 12, 7, and 8 CSPs were identified in *D. ponderosae*, *I. typographus M. alternatus*, *D. helophoroides,* and *R. dominica,* respectively (Andersson et al., 2013; Wang et al., 2014; Diakite et al., 2015). The number of CSP genes in *L. decemlineata* we identified in this study is comparable with previous reports on these five beetles.

SNMPs were first identified in pheromone-sensitive neurons of Lepidopteran (Rogers et al., 2001) and are thought to play a role in pheromone detection (Benton et al., 2007). There are two families of SNMPs (SNMP1 and SNMP2) identified in most insects including Lepidopteran and Dipteran (Liu et al., 2012; Cao et al., 2014). But in the transcriptome of *D. ponderosae*

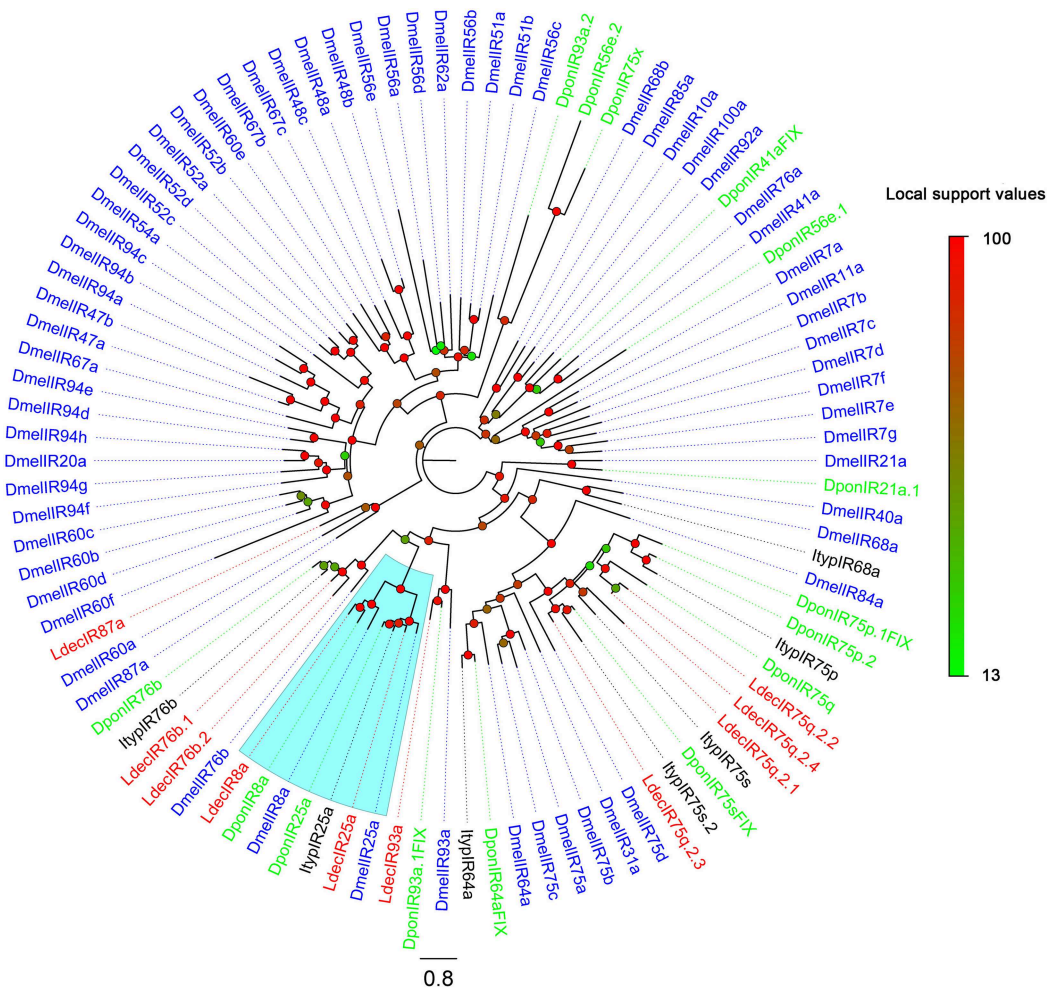

FIGURE 6 | Phylogenetic tree of candidate LdecIRs with IRs from other insects. Dpon, *D. ponderosae*; Ityp, *I. typographus*; *Dmel*, *D. melanogaster*. The clade in blue indicates the IR8a/IR25a clade.

and *I. typographus*, there are three SNMPs identified (Andersson et al., 2013). We also found three SNMPs (LdecSNMP1-3) in our transcriptome.

A total of 37 OR genes were identified within the *L. decemlineata* antennal transcriptome. In the genome of *T. castaneum*, a total of 239 genes coding candidate ORs were detected (Richards et al., 2008; Kim et al., 2010), that is much more than ORs identified in other insect genomes including *D. melanogaster* (62) (Adams et al., 2000), *B. mori* (64) (Tanaka et al., 2009), *A. gambiae* (79) (Fox et al., 2001), and *Apis mellifera* (170) (Robertson and Wanner, 2006). Without genomic information, the ORs identified by transcriptome analysis were usually much less., likely due to some ORs don't express in antennae of adult. The number of ORs identified in *M. caryae* (Mitchell et al., 2012), *I. typographus* (Andersson et al., 2013) and *D. ponderosae* (Andersson et al., 2013) were 57, 43, and 49, respectively, which was higher than *L. decemlineata*. The lengths of candidate ORs in *L. decemlineata* was also substandard, despite the fact that, the sequencing depth of our transcriptome was even greater than other three Coleopteran transcriptomes. Furthermore, the numbers of OBPs and CSPs identified in our study were at comparable level or even much higher than other three. These all suggest a high quantity of our transcriptome sequencing. There are two possibilities to address the phenomena of relatively fewer candidate OR genes in *L. decemlineata* antennal transcriptome. First, the number of ORs in *L. decemlineata* is actually less than other species. Second, the expression level of ORs in *L. decemlineata* antenna is very low, resulting in lower detection metrics. The low expression level of LdecORs was further shown by the RT-PCR experiments.

Most of the candidate OR genes have similar expression level in male and female based on RT-PCR detection. In previous studies, male-produced aggregation pheromone has been identified, and both male and female Colorado potato beetles could be attracted (Dickens et al., 2002). The male and female adults could also be attracted by odors released by host plants (de Wilde et al., 1969). The consistently expressed ORs might be involved in these behaviors. A few OR genes have been detected in RT-PCR as having male-specific (*LdecOR6*) or male-biased (*LdecOR5*, *LdecOR12*, *LdecOR26,* and *LdecOR32*)

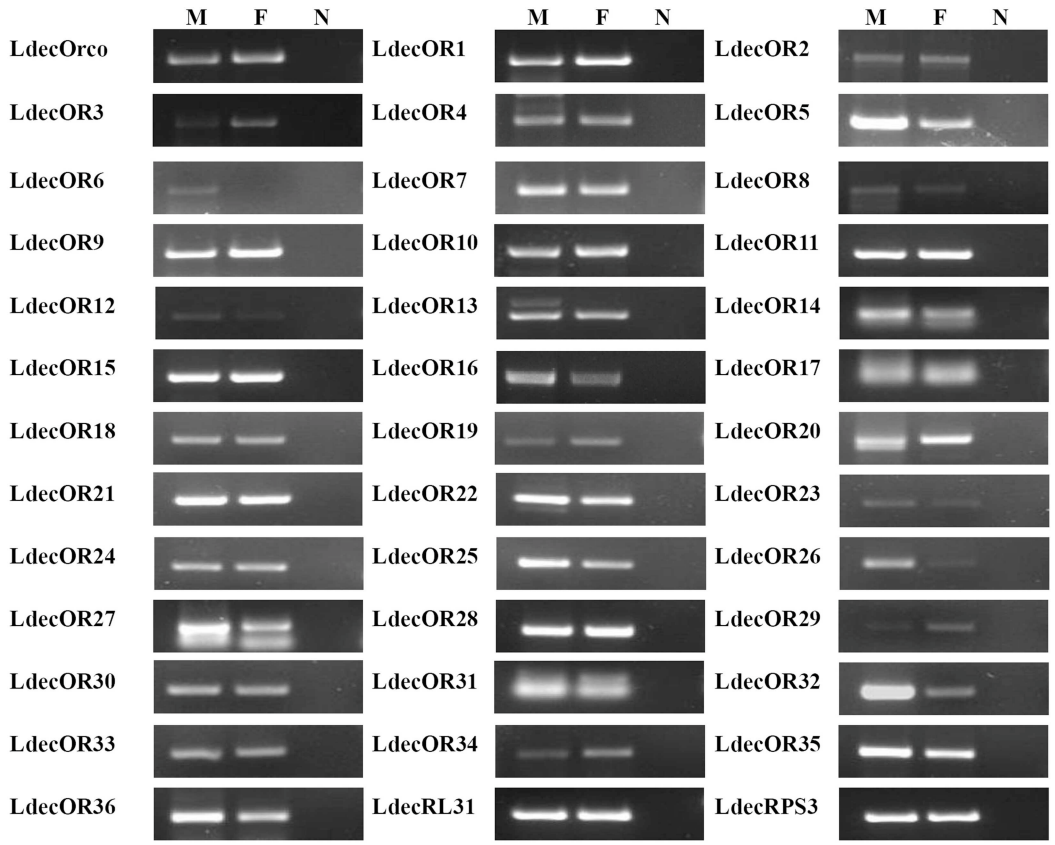

FIGURE 7 | Sex-specific expressions of candidates *LdecORs*. M, male antennae; F, female antennae; N: negative control.

expression, and may take part in the detection of the sex pheromone or other male-specific behaviors. On the other hand, *LdecOR3* and *LdecOR29* were observed to be expressed higher in female, which suggested they might participate in female-specific behaviors such as oviposition site selection.

In this study, ten IR candidates including two co-receptors, IR8a and IR25a were annotated in *L. decemlineata* antennal transcriptome. Compared with ORs, the sequences IRs are relatively conserved. Among the ten LdecIRs, nine sequences have orthologs in *I. typographus* and *D. ponderosae* (Andersson et al., 2013). The potential ortholog of LdecIR87a was also found in *D. melanogaster* (Benton et al., 2009). Considering the relatively high sequence conservation, the functions of IRs are probably conserved among Coleoptera.

Conclusions

The main objective of antennal transcriptome sequencing was to identify genes potentially involved in olfactory signal detection in *L. decemlineata*. The number of IRs, OBPS, CSPs, and SNMPs identified in this species is close to the complete repertoire of olfactory system genes identified from other Coleopteran species. The number of ORs in *L. decemlineata* appeared to be lower than other Coleopterans. This might be the result of the low expression level of ORs which has been confirmed by RT-PCR. Our findings lay the foundation for future research on the molecular basis of olfactory system of *L. decemlineata* and provide information for comparative and functional genomic analyses of Coleopteran species.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2015.00060/abstract

Supplementary Material S1 | Accession numbers for amino acid sequences of ORs, IRs, OBPs, and CSPs used in phylogenetic analyses (xlsx).

Supplementary Material S2 | Primers for RT-PCR expression analyses of *L. decemlineata* ORs (xlsx).

Supplementary Material S3 | Amino acid sequences of candidate olfactory genes identified in this study, FASTA formatted file (fasta).

References

Abuin, L., Bargeton, B., Ulbrich, M. H., Isacoff, E. Y., Kellenberger, S., and Benton, R. (2011). Functional architecture of olfactory ionotropic glutamate receptors. *Neuron* 69, 44–60. doi: 10.1016/j.neuron.2010.11.042

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., et al. (2000). The genome sequence of *Drosophila melanogaster*. *Science* 287, 2185–2195. doi: 10.1126/science.287.5461.2185

Ai, M. R., Blais, S., Park, J. Y., Min, S., Neubert, T. A., and Suh, G. S. B. (2013). Ionotropic glutamate receptors IR64a and IR8a form a functional odorant receptor complex *in vivo* in *Drosophila*. *J. Neurosci.* 33, 10741–10749. doi: 10.1523/JNEUROSCI.5419-12.2013

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402. doi: 10.1093/nar/25.17.3389

Andersson, M. N., Grosse-Wilde, E., Keeling, C. I., Bengtsson, J. M., Yuen, M. M. S., Li, M., et al. (2013). Antennal transcriptome analysis of the chemosensory gene families in the tree killing bark beetles, *Ips typographus* and *Dendroctonus ponderosae* (Coleoptera: curculionidae: scolytinae). *BMC Genomics* 14:198. doi: 10.1186/1471-2164-14-198

Bengtsson, J. M., Trona, F., Montagne, N., Anfora, G., Ignell, R., Witzgall, P., et al. (2012). Putative chemosensory receptors of the codling moth, *Cydia pomonella*, identified by antennal transcriptome analysis. *PLoS ONE* 7:e31620. doi: 10.1371/journal.pone.0031620

Benton, R., Sachse, S., Michnick, S. W., and Vosshall, L. B. (2006). Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors *in vivo*. *PLoS Biol.* 4:e20. doi: 10.1371/journal.pbio.0040020

Benton, R., Vannice, K. S., Gomez-Diaz, C., and Vosshall, L. B. (2009). Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. *Cell* 136, 149–162. doi: 10.1016/j.cell.2008.12.001

Benton, R., Vannice, K. S., and Vosshall, L. B. (2007). An essential role for a CD36-related receptor in pheromone detection in *Drosophila*. *Nature* 450, 289–293. doi: 10.1038/nature06328

Cao, D., Liu, Y., Wei, J., Liao, X., Walker, W. B., Li, J., et al. (2014). Identification of Candidate olfactory genes in *Chilo suppressalis* by antennal transcriptome analysis. *Int. J. Biol. Sci.* 10, 846–860. doi: 10.7150/ijbs.9297

Clyne, P. J., Warr, C. G., Freeman, M. R., Lessing, D., Kim, J., and Carlson, J. R. (1999). A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron* 22, 327–338. doi: 10.1016/S0896-6273(00)81093-4

Conesa, A., Gotz, S., Garcia-Gomez, J. M., Terol, J., Talon, M., and Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21, 3674–3676. doi: 10.1093/bioinformatics/bti610

Croset, V., Rytz, R., Cummins, S. F., Budd, A., Brawand, D., Kaessmann, H., et al. (2010). Ancient protostome origin of chemosensory ionotropic glutamate receptors and the evolution of insect taste and olfaction. *PLoS Genet* 6:e1001064. doi: 10.1371/journal.pgen.1001064

de Bruyne, M., and Baker, T. (2008). Odor detection in insects: volatile codes. *J. Chem. Ecol.* 34, 882–897. doi: 10.1007/s10886-008-9485-4

de Wilde, J., Hille Ris Lambers-Suverkropp, K., and van Tol, A. (1969). Responses to air flow and airborne plant odour in the Colorado beetle. *Neth. J. Plant Pathol.* 75, 53–57. doi: 10.1007/BF02137193

Diakite, M. M., Wang, J., Ali, S., and Wang, M. Q. (2015). Identification of chemosensory gene families in Rhyzopertha dominica (Coleoptera Bostrichidae). *Can. Entomol.* doi: 10.4039/tce.2015.13. (in press).

Dickens, J. C., Oliver, J. E., Hollister, B., Davis, J. C., and Klun, J. A. (2002). Breaking a paradigm: male-produced aggregation pheromone for the Colorado potato beetle. *J. Exp. Biol.* 205, 1925–1933.

Foret, S., Wanner, K. W., and Maleszka, R. (2007). Chemosensory proteins in the honey bee: Insights from the annotated genome, comparative analyses and expressional profiling. *Insect Biochem. Mol. Biol.* 37, 19–28. doi: 10.1016/j.ibmb.2006.09.009

Fox, A. N., Pitts, R. J., Robertson, H. M., Carlson, J. R., and Zwiebel, L. J. (2001). Candidate odorant receptors from the malaria vector mosquito *Anopheles gambiae* and evidence of down-regulation in response to blood feeding. *Proc. Natl. Acad. Sci. U.S.A.* 98, 14693–14697. doi: 10.1073/pnas.261432998

Gong, D. P., Zhang, H. J., Zhao, P., Xia, Q. Y., and Xiang, Z. H. (2009). The odorant binding protein gene family from the genome of silkworm, *Bombyx mori*. *BMC Genomics* 10:332. doi: 10.1186/1471-2164-10-332

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* 29, 644–652. doi: 10.1038/nbt.1883

Grosse-Wilde, E., Kuebler, L. S., Bucks, S., Vogel, H., Wicher, D., and Hansson, B. S. (2011). Antennal transcriptome of *Manduca sexta*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 7449–7454. doi: 10.1073/pnas.1017963108

Hekmat-Scafe, D. S., Scafe, C. R., McKinney, A. J., and Tanouye, M. A. (2002). Genome-wide analysis of the odorant-binding protein gene family in *Drosophila melanogaster*. *Genome. Res.* 12, 1357–1369. doi: 10.1101/gr.239402

Hildebrand, J. G. (1995). Analysis of Chemical Signals by Nervous Systems. *Proc. Natl. Acad. Sci. U.S.A.* 92, 67–74. doi: 10.1073/pnas.92.1.67

Hildebrand, J. G., and Shepherd, G. M. (1997). Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. *Annu. Rev. Neurosci.* 20, 595–631. doi: 10.1146/annurev.neuro.20.1.595

Hunt, T., Bergsten, J., Levkanicova, Z., Papadopoulou, A., John, O. S., Wild, R., et al. (2007). A comprehensive phylogeny of beetles reveals the evolutionary origins of a superradiation. *Science* 318, 1913–1916. doi: 10.1126/science.1146954

Jacquin-Joly, E., Legeai, F., Montagne, N., Monsempes, C., Francois, M. C., Poulain, J., et al. (2012). Candidate chemosensory genes in female antennae of the noctuid moth *Spodoptera littoralis*. *Int. J. Biol. Sci.* 8, 1036–1050. doi: 10.7150/ijbs.4469

Jones, P. L., Pask, G. M., Rinker, D. C., and Zwiebel, L. J. (2011). Functional agonism of insect odorant receptor ion channels. *Proc. Natl. Acad. Sci. U.S.A.* 108, 8821–8825. doi: 10.1073/pnas.1102425108

Kaissling, K.-E., and Colbow, K. (1987). *RH Wright Lectures on Insect Olfaction*. British Columbia: Simon Fraser University Burnaby.

Kanaujia, S., and Kaissling, K. (1985). Interactions of pheromone with moth antennae: adsorption, desorption and transport. *J. Insect Physiol.* 31, 71–81. doi: 10.1016/0022-1910(85)90044-7

Katoh, K., and Toh, H. (2008). Recent developments in the MAFFT multiple sequence alignment program. *Brief. Bioinform.* 9, 286–298. doi: 10.1093/bib/bbn013

Kim, H. S., Murphy, T., Xia, J., Caragea, D., Park, Y., Beeman, R. W., et al. (2010). BeetleBase in 2010: revisions to provide comprehensive genomic information for *Tribolium castaneum*. *Nucleic Acids Res.* 38, D437–D442. doi: 10.1093/nar/gkp807

Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* 305, 567–580. doi: 10.1006/jmbi.2000.4315

Kuhar, T., Mori, K., and Dickens, J. (2006). Potential of a synthetic aggregation pheromone for integrated pest management of Colorado potato beetle. *Agr. Forest. Entomol.* 8, 77–81. doi: 10.1111/j.1461-9555.2006.00286.x

Larsson, M. C., Domingos, A. I., Jones, W. D., Chiappe, M. E., Amrein, H., and Vosshall, L. B. (2004). Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* 43, 703–714. doi: 10.1016/j.neuron.2004.08.019

Laughlin, J. D., Ha, T. S., Jones, D. N., and Smith, D. P. (2008). Activation of pheromone-sensitive neurons is mediated by conformational activation of pheromone-binding protein. *Cell* 133, 1255–1265. doi: 10.1016/j.cell.2008.04.046

Legeai, F., Malpel, S., Montagné, N., Monsempes, C., Cousserans, F., Merlin, C., et al. (2011). An Expressed Sequence Tag collection from the male antennae of the Noctuid moth *Spodoptera littoralis*: a resource for olfactory and pheromone detection research. *BMC Genomics* 12:86. doi: 10.1186/1471-2164-12-86

Liu, Y., Gu, S., Zhang, Y., Guo, Y., and Wang, G. (2012). Candidate olfaction genes identified within the *Helicoverpa armigera* antennal transcriptome. *PLoS ONE* 7:e48260. doi: 10.1371/journal.pone.0048260

Min, X. J., Butler, G., Storms, R., and Tsang, A. (2005). OrfPredictor: predicting protein-coding regions in EST-derived sequences. *Nucleic Acids Res.* 33, W677–W680. doi: 10.1093/nar/gki394

Mitchell, R. F., Hughes, D. T., Luetje, C. W., Millar, J. G., Soriano-Agaton, F., Hanks, L. M., et al. (2012). Sequencing and characterizing odorant receptors of the cerambycid beetle *Megacyllene caryae*. *Insect Biochem. Mol. Biol.* 42, 499–505. doi: 10.1016/j.ibmb.2012.03.007

Mustaparta, H. (1990). Chemical information processing in the olfactory system of insects. *Physiol. Rev.* 70, 199–245.

Neuhaus, E. M., Gisselmann, G., Zhang, W., Dooley, R., Stortkuhl, K., and Hatt, H. (2005). Odorant receptor heterodimerization in the olfactory system of *Drosophila melanogaster*. *Nat. Neurosci.* 8, 15–17. doi: 10.1038/nn1371

Pelosi, P., and Maida, R. (1995). Odorant-binding proteins in insects. *Comp. Biochem. Physiol. B.* 111, 503–514. doi: 10.1016/0305-0491(95)00019-5

Pertea, G., Huang, X., Liang, F., Antonescu, V., Sultana, R., Karamycheva, S., et al. (2003). TIGR gene indices clustering tools (TGICL): a software system for fast clustering of large EST datasets. *Bioinformatics* 19, 651–652. doi: 10.1093/bioinformatics/btg034

Petersen, T. N., Brunak, S., von Heijne, G., and Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* 8, 785–786. doi: 10.1038/nmeth.1701

Poivet, E., Gallot, A., Montagné, N., Glaser, N., Legeai, F., and Jacquin-Joly, E. (2013). A comparison of the olfactory gene repertoires of adults and larvae in the noctuid moth *Spodoptera littoralis*. *PloS ONE* 8:e60263. doi: 10.1371/journal.pone.0060263

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2–approximately maximum-likelihood trees for large alignments. *PLoS ONE* 5:e9490. doi: 10.1371/journal.pone.0009490

Richards, S., Gibbs, R. A., Weinstock, G. M., Brown, S. J., Denell, R., Beeman, R. W., et al. (2008). The genome of the model beetle and pest *Tribolium castaneum*. *Nature* 452, 949–955. doi: 10.1038/nature06784

Robertson, H. M., and Wanner, K. W. (2006). The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. *Genome. Res.* 16, 1395–1403. doi: 10.1101/gr.5057506

Rogers, M. E., Krieger, J., and Vogt, R. G. (2001). Antennal SNMPs (sensory neuron membrane proteins) of Lepidoptera define a unique family of invertebrate CD36-like proteins. *J. Neurobiol.* 49, 47–61. doi: 10.1002/neu.1065

Rützler, M., and Zwiebel, L. J. (2005). Molecular biology of insect olfaction:recent progress and conceptual models. *J. Comp. Physiol. A* 191, 777–790. doi: 10.1007/s00359-005-0044-y

Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L. B., and Touhara, K. (2008). Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature* 452, 1002–1006. doi: 10.1038/nature06850

Sato, K., and Touhara, K. (2009). "Insect olfaction: receptors, signal transduction, and behavior," in *Chemosensory Systems in Mammals, Fishes, and Insects*, eds S. Korsching and W. Meyerhof (Berlin; Heidelberg: Springer), 203–220. doi: 10.1007/400_2008_10

Shanbhag, S. R., Muller, B., and Steinbrecht, R. A. (1999). Atlas of olfactory organs of Drosophila melanogaster—1. Types, external organization, innervation and distribution of olfactory *sensilla*. *Int. J. Insect. Morphol.* 28, 377–397. doi: 10.1016/S0020-7322(99)00039-2

Shanbhag, S. R., Muller, B., and Steinbrecht, R. A. (2000). Atlas of olfactory organs of *Drosophila melanogaster* 2. Internal organization and cellular architecture of olfactory *sensilla*. *Arthropod. Struct. Dev.* 29, 211–229. doi: 10.1016/S1467-8039(00)00028-1

Tanaka, K., Uda, Y., Ono, Y., Nakagawa, T., Suwa, M., Yamaoka, R., et al. (2009). Highly selective tuning of a silkworm olfactory receptor to a key mulberry leaf volatile. *Curr. Biol.* 19, 881–890. doi: 10.1016/j.cub.2009.04.035

Vogt, R. G., Miller, N. E., Litvack, R., Fandino, R. A., Sparks, J., Staples, J., et al. (2009). The insect SNMP gene family. *Insect Biochem. Mol. Biol.* 39, 448–456. doi: 10.1016/j.ibmb.2009.03.007

Wang, J., Li, D. Z., Min, S. F., Mi, F., Zhou, S. S., and Wang, M. Q. (2014). Analysis of chemosensory gene families in the beetle *Monochamus alternatus* and its parasitoid *Dastarcus helophoroides*. *Comp. Biochem. Physiol. Part D Genomics Proteomics* 11, 1–8. doi: 10.1016/j.cbd.2014.05.001

Wicher, D., Schäfer, R., Bauernfeind, R., Stensmyr, M. C., Heller, R., Heinemann, S. H., et al. (2008). *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature* 452, 1007–1011. doi: 10.1038/nature06861

Wojtasek, H., and Leal, W. S. (1999). Conformational change in the pheromone-binding protein from *Bombyx mori* induced by pH and by interaction with membranes. *J. Biol. Chem.* 274, 30950–30956. doi: 10.1074/jbc.274.43.30950

Xia, Q., Zhou, Z., Lu, C., Cheng, D., Dai, F., Li, B., et al. (2004). A draft sequence for the genome of the domesticated silkworm (*Bombyx mori*). *Science* 306, 1937–1940. doi: 10.1126/science.1102210

Xu, P. X., Atkinson, R., Jones, D. N. M., and Smith, D. P. (2005). *Drosophila* OBP LUSH is required for activity of pheromone-sensitive neurons. *Neuron* 45, 193–200. doi: 10.1016/j.neuron.2004.12.031

Zhou, J. J. (2010). Odorant-binding proteins in insects. *Vitam. Horm.* 83, 241–272. doi: 10.1016/S0083-6729(10)83010-9

Zhou, J. J., He, X. L., Pickett, J. A., and Field, L. M. (2008). Identification of odorant-binding proteins of the yellow fever mosquito *Aedes aegypti*: genome annotation and comparative analyses. *Insect Mol. Biol.* 17, 147–163. doi: 10.1111/j.1365-2583.2007.00789.x

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METHODS
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Protocol for Heterologous Expression of Insect Odourant Receptors in *Drosophila*

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Insect olfactory receptors (ORs) are tuned to volatile chemicals, they are expressed in the membrane of olfactory sensory neurons (OSNs), housed in sensilla on the antenna. The olfactory apparatus is under strong selection and ORs are tuned to vital chemical signals, mediating social communication, feeding and oviposition, and avoidance of predators and pathogens. An emerging technique to reliably and efficiently identify the key ligands of ORs is to express single ORs in heterologous cell systems for subsequent screening. Several *in vivo* and *in vitro* platforms have been developed; we here provide a step-by-step protocol for OR expression in *Drosophila melanogaster* OSNs. Following RNA extraction, molecular cloning of ORs and injection of plasmid vectors into *Drosophila* embryos to create flies with OR transgenes, single ORs are expressed, via crossing with specific transgene promoters in OSNs of ab3 and T1 antennal sensilla. This approach enables replicable single sensillum electrophysiological recordings (SSR) from readily distinguishable *Drosophila* sensilla, containing OSNs expressing transgenic ORs. We expect this method to be applicable to ORs across insect orders and to increasingly contribute to chemical ecology research. Heterologous expression enables thorough investigation of single ORs, toward the identification of yet unknown, behaviorally and ecologically relevant chemical signals. It also enables investigations of the functional properties of ORs and their evolutionary diversification, through comparative structure-activity studies across phylogenies.

Keywords: insect ORs, heterologous expression, empty neuron system, single sensillum recordings, deorphanization

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INTRODUCTION

Olfactory communication signals are recruited from countless volatile chemicals filling the air. A foremost goal in insect chemical ecology research is to unambiguously identify behavior-modifying compounds, termed semiochemicals, which convey messages from animals, plants or microbes. Semiochemicals usually are blends of several compounds and it is a sensitive and time-consuming task to discriminate between behaviorally active and inactive compounds found in headspace collections.

In insects, electrophysiological recordings, which employ the antenna as sensor, have been a versatile and widely used tool for selecting candidate compounds (Schneider, 1957; Arn et al., 1975) and facilitate interlacing chemical with behavioral analysis. Recordings from entire antennae

are particularly efficient for identification of sex pheromones, used for communication within the same species, and typically elicit a conspicuous response. Knowledge of *de-novo* produced pheromones also facilitates further identifications, since taxonomically close species use related biosynthetic pathways (Jurenka, 2004). Consequently, hundreds of lepidopteran pheromones have been described (Arn et al., 1992; El-Sayed, 2015).

In comparison, unequivocal identification of kairomones, compounds which guide host plant attraction, in moths and other herbivorous insects is infinitely more difficult. Plants release a wealth of compounds and, in contrast with sex pheromones, there is no producer-receiver correlation—abundance of plant compounds is no criterium for behavioral activity. Plant volatiles that attract herbivores have long been known (Dethier, 1947; El-Sayed, 2015), but we still do not know as to whether, or to what extent these attractants actually correspond to the chemical signatures used by insects to find their host plants. The attractant power of synthetic kairomones is a straightforward criterium, but behavioral assays with kairomones, especially in females, are complex and laborious.

Screening candidate compounds prior to behavioral analysis is therefore paramount. Unfortunately, for the identification of kairomones, conventional antennal electrophysiological recordings fail to deliver. The most abundant compounds in plant headspace invariably produce a response when recording from the entire antenna, disregarding their behavioral relevance. Recordings from single olfactory sensilla, on the other hand, are technically demanding and will only rarely provide exhaustive information. This is exemplified by work on codling moth, where the main apple volatiles produce a strong antennal, but only weak or no behavioral response (Bäckman et al., 2001; Ansebo et al., 2004; Coracini et al., 2004). In contrast, pear ester, a compound which has not been found in the main host apple, is the strongest known adult and larval attractant (Light et al., 2001; Light and Knight, 2005; Light and Beck, 2012).

Following the identification of olfactory receptor (OR) genes from codling moth antennae (Bengtsson et al., 2012), it has recently been shown that CpomOR3, which is highly expressed in male and female antennae, is specifically tuned to pear ester (Bengtsson et al., 2014). This finding corroborates the biological role of pear ester and is supported by intracellular recordings and functional imaging of the codling moth antennal lobe (Trona et al., 2010, 2013). The functional characterization of CpomOR3 also underscores the weight of a reliable screening technique for single ORs—toward a more efficient identification of semiochemicals of plant origin.

In silico identification of putative odourant receptor (OR) genes in *Drosophila melanogaster* was the starting point for a new era of chemical communication research and opened the door for downstream studies in which ORs are functionally characterized according to the ligands they are tuned to, a process also known as "deorphanization" (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999; Hallem et al., 2004; Hallem and Carlson, 2006). Deorphanization of insect ORs is achieved through testing their response spectrum toward odourant compounds, following heterologous expression of these OR proteins in heterospecific cell systems, which facilitates thorough and unambigious screening.

In vitro systems involve the expression of ORs in cell culture platforms, such as human embryonic kidney cells (HEK; Große-Wilde et al., 2006; Syed et al., 2006; Corcoran et al., 2014), *Spodoptera frugiperda* Sf9 cells (Matarazzo et al., 2005; Kiely et al., 2007; Anderson et al., 2009; Jordan et al., 2009; Xu et al., 2015) and also *Xenopus* oocytes (Sakurai et al., 2004; Mitsuno et al., 2008; Wanner et al., 2010; Leary et al., 2012; Liu et al., 2013; Zhang and Löfstedt, 2013; Jiang et al., 2014). Recently, a cell-free expression system has been reported (Tegler et al., 2015).

In the case of *in vivo* systems, heterologous expression is based on the use of mutant, "empty-neuron" lines of *D. melanogaster* (Dobritsa et al., 2003; Hallem et al., 2004). The antennal basiconic sensilla type 3 (ab3) of the mutant *D. melanogaster* flies contain an odourant sensory neuron (OSN) that lacks its native OR: expression of the native OR22a/b in ab3A OSNs is disrupted in these mutant flies (Dobritsa et al., 2003). When coupled with the Gal4-UAS transgene expression system (Brand and Perrimon, 1993), using an OR22a Gal4 line, transgenic ORs can be specifically expressed in ab3A empty OSNs, which project their dendrites into large basiconic sensilla (Shanbhag et al., 1999). These OSNs can then be screened for novel responses conferred by the transgenic OR, by means of single sensillum electrophysiological recordings (SSR). This methodology has been successful for the deorphanization of receptors from different subsystems such as antennal ORs as well as maxillary palp ORs (Dobritsa et al., 2003; Goldman et al., 2005). In addition, the empty neuron system has also allowed to deorphanize larval receptors (Kreher et al., 2005, 2008; Mathew et al., 2013).

Deorphanization of putative pheromone receptors (PRs) has proven to be more challenging than OR deorphanization. To provide PRs with a more suitable cellular environment, heterologous expression has instead targeted the trichoid sensillum T1 of *D. melanogaster*. In wild-type flies, T1 sensilla contain a single neuron expressing a single receptor, OR67d, which is tuned to the male pheromone, 11-*cis*-vaccenyl acetate (cVA). In knock-in mutant flies, this native receptor is replaced with an OR67d-Gal4 construct (Kurtovic et al., 2007). The T1 system is suitable for the deorphanization of both PRs (Syed et al., 2010; Montagné et al., 2012) and some ORs tuned to plant compounds (Bengtsson et al., 2014; Ronderos et al., 2014).

Heterologous expression in *Drosophila* has served as a fundamental tool for the deorphanization of insect ORs and PRs across diverse taxa. However, the procedures necessary to produce flies expressing transgenic receptors have not been comprehensively described. Here, we provide a hands-on, step-by-step protocol of how to express and test insect ORs in Drosophila OSNs.

MATERIALS AND EQUIPMENT

Reagents and materials required for the different steps in producing and testing transgenic fly lines that ectopically express ORs in the empty neuron systems are shown in **Table 1**.

TABLE 1 | Materials and equipment.

Protocol	Step	Materials	Equipment	Notes
Molecular cloning of insect ORs	RNA extraction and purification	Dissected target insect tissue		
		RNA extraction/purification kit		
	First strand cDNA synthesis	Purified RNA sample	Thermocycler/incubator machine	
		First strand cDNA synthesis kit		
	PCR amplification of target OR sequence	Target tissue cDNA sample	Thermocycler/incubator machine	
		Ultra-pure molecular biology grade sterile water		
		Proofreading *Taq* polymerase and 10× polymerase buffer		
		Gene specific forward and reverse PCR primers (10 μM)		
		dNTPs (10 mM)		
	Gel analysis of PCR product and gel purification	Molecular grade agarose Deionized water DNA intercalating agent Razor blades	UV light source/agarose gel imaging system	
		Standard DNA gel extraction kit	Electric heating block apparatus	
	TOPO gateway cloning of target OR sequence and confirmation of desired construct	Gel-purified PCR product	Thermocycler/incubator machine	Catalog No. K2500-20 (Thermo Fisher Scientific)
		PCR-8/GW/TOPO TA cloning kit with *E. coli* bacteria		
		Ultra-pure molecular biology grade sterile water		
		Non-proofreading *Taq* polymerase and 10× buffer reagents		
		dNTPs (10 mM)		CAS No. 22189-32-8
		Spectinomycin Antibiotic solution (50 micrograms per mL) with Spectinomycin dihydrochloride pentahydrate		
		LB Agar Powder		
		LB Medium Powder		
		TOPO-GW plasmid forward (GW1) and reverse (GW2) PCR/sequencing primers (10 μM)		GW1 sequence: GTTGCAACAAATTGATGAGCAATGC
		OR gene specific forward (GSP1) and reverse (GSP2) PCR primers (10 μM)		GW2 sequence: GTTGCAACAAATTGATGAGCAATTA
		Standard plasmid mini-prep purification kit		
	Clonase transfer of OR insert to Gateway destination vector	TOPO-GW plasmid with OR insert (25 ng/μL) pUASg.attB destination plasmid LR Clonase II enzyme mix kit with proteinase K solution	Thermocycler-incubator machine Standard laboratory incubator shaker	Destination plasmid obtained from the Basler lab FlyC31 website (http://www.flyc31.org/)
		Ampicillin antibiotic solution (50 μg/mL) with ampicillin sodium salt		Catalog No. 11791-020 (Thermo Fisher Scientific)
		LB agar powder LB medium powder	Standard laboratory growth chamber incubator	CAS No. 69-52-3
		Plasmid mini-prep purification kit pUASg.attB plasmid forward (UAS1) and reverse (UAS2) sequencing primers (each 10 μM)		UAS1 sequence: TAGCGAGCGCCGGAGTATAAATAG UAS2 sequence: ACTGATTTCGACGGTTACCC
Transgenic expression of ORs in empty neuron system	Genetic crosses	Transgenic fly strains with red/orange eye color		Presumes the laboratory maintains or has access to fly rearing facilities. The fly-lines indicated below are available upon request
		Double balancer Bl/Cyo; TM2/TM6b fly line		
		ΔHalo/Cyo; TM2/TM6b fly line		
		ΔHalo/Cyo; DmelOR22a-Gal4 fly line		
		w−; Bl/Cyo; DmelOR67d-Gal4 fly line		

(Continued)

TABLE 1 | Continued

Protocol	Step	Materials	Equipment	Notes
Single sensillum recordings	Mounting	200-μl Pipette tips Full-length glass microscope slide Piece of microscope slide (one fifth of full-length) Glass capillary Dental wax		
	Recordings	2 Sharpened tungsten electrodes (0.1 mm Ø)	2 Electrode holders: for reference and for recording electrode DC-3K Micromanipulator equipped with a PM-10 piezo translator INR-02 Probe Channel USB signal acquisition controller (IDAC-4) Stimulus controller Software for visualization and analysis (Autospike)	
	Odourant stimulation	Glass Pasteur pipettes Filter paper (1.5 × 1 cm) Solvent (hexane, paraffin oil, ethanol, acetone, or other) Diagnostic compounds diluted in the selected solvent at maximum dose of 1 μg/μl. For ab3A empty neuron system: ethyl-3-hydroxybutyrate, 2-heptanone, ethyl hexanoate or ethyl butyrate. For T1 empty neuron system: cVA		

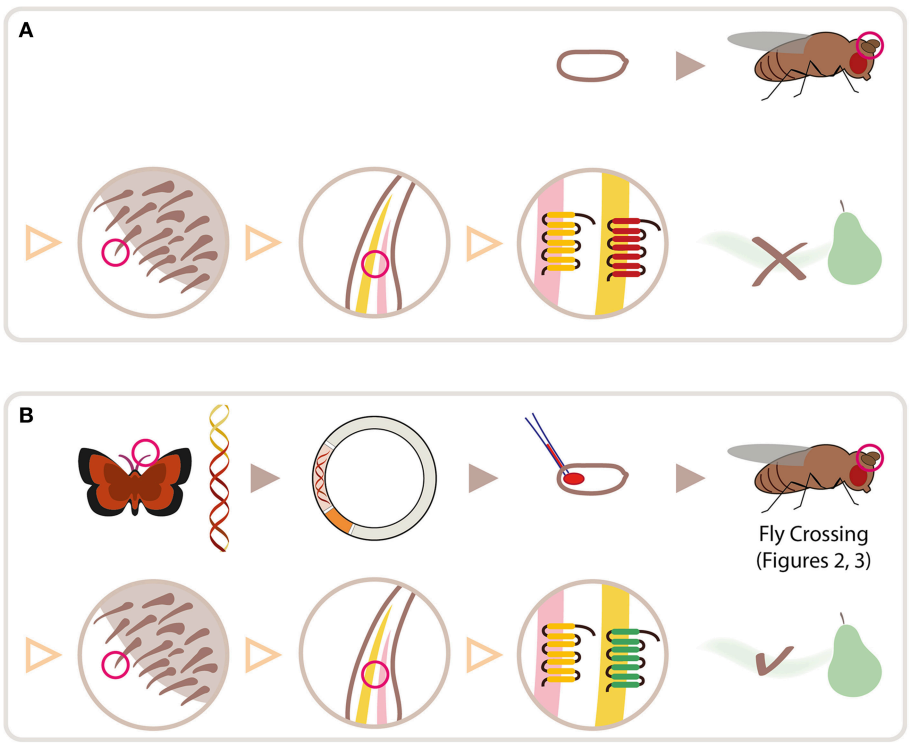

FIGURE 1 | Schematic overview for heterologous expression of insect ORs in *Drosophila* OSNs. (A) Wild type fly embryo and fly (top row). Red circle highlights antenna, shown in three magnification steps (second row, separated by triangles): sensilla on antenna; 2 olfactory sensory neurons (OSNs) housed in one sensillum; olfactory receptor proteins (ORs) expressed in cell membrane of each OSN. Wild type flies do not smell pear ester. **(B)** cDNA is synthesized from RNA extracted from lepidopteran antennae; OR gene is cloned into plasmid; plasmid is injected into fly embryo. Following crosses using Gal4/UAS expression system, lepidopteran OR tuned to pear ester is expressed in target OSN on fly antenna, allowing it to detect pear ester. Moth and fly drawing by Katarina Eriksson (www.markadesign.se).

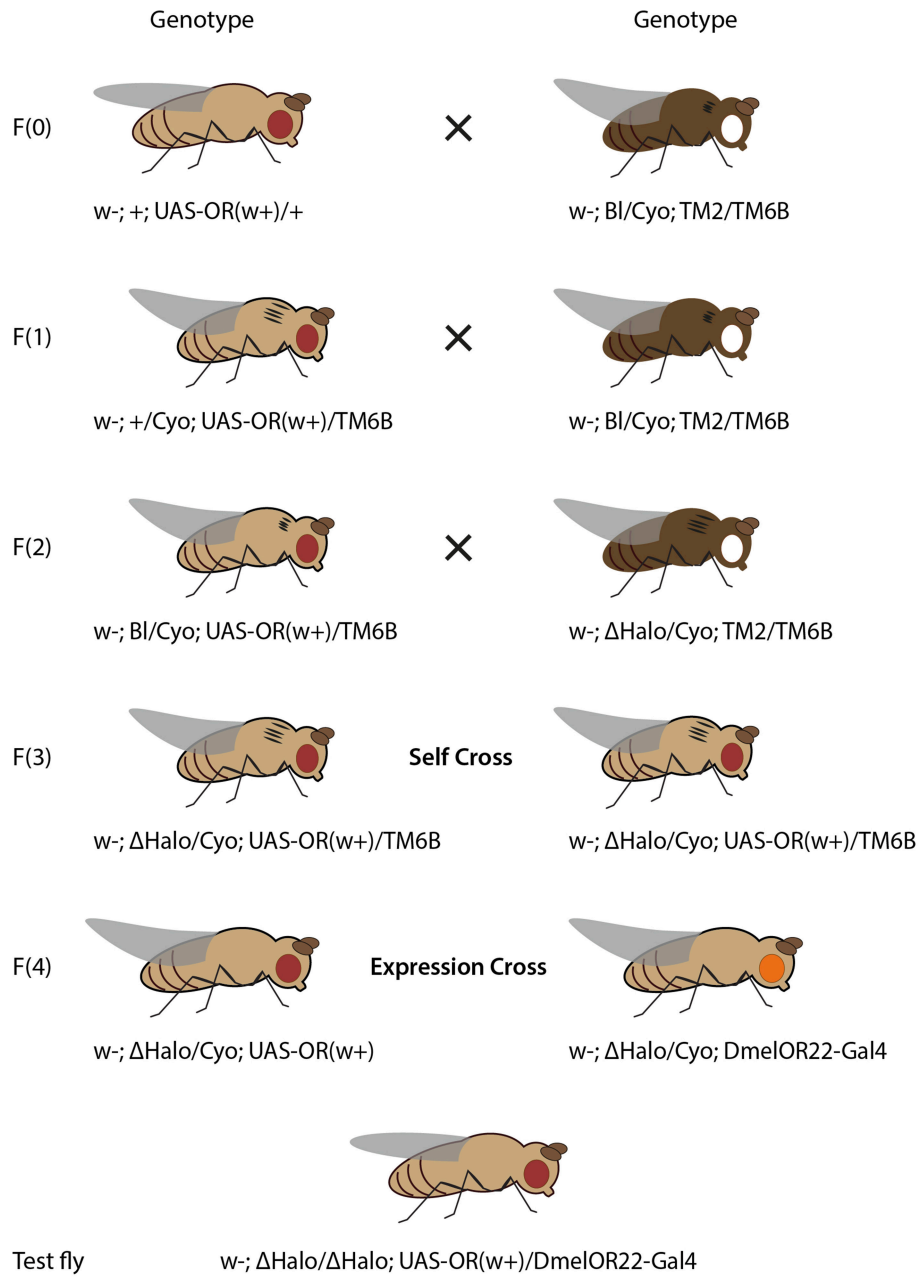

FIGURE 2 | Crossing scheme for heterologous expression of an OR transgene in empty neurons in ab3 sensilla, using ΔHalo mutant background and DmelOR22a-Gal4 driver line. Fly drawing by Katarina Eriksson (www.markadesign.se).

PROCEDURES

For efficient streamlined cloning of OR genes and generation of transgenic flies, we recommend use of the TOPO/gateway cloning system (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) coupled to PhiC31 integrase-mediated transgenesis system applied to *D. melanogaster* (Bischof et al., 2007). The TOPO/gateway system facilitates cloning and transfer of DNA inserts from entry to destination plasmid and the Phi3C31 system facilitates highly-efficient, non-random, sequence-directed and irreversible genomic insertion of vector DNA. The following protocols have been formulated specifically for use of these systems. Whether the goal is to express an OR transgene in the ab3A or T1 systems, the molecular cloning procedures in the following section are identical up until the point of embryonic injections, as described below.

An overview of the two main worksteps, molecular cloning (Section Molecular Cloning of Insect ORs) and transgenic expression by fly crossing (Section Transgenic Expression of

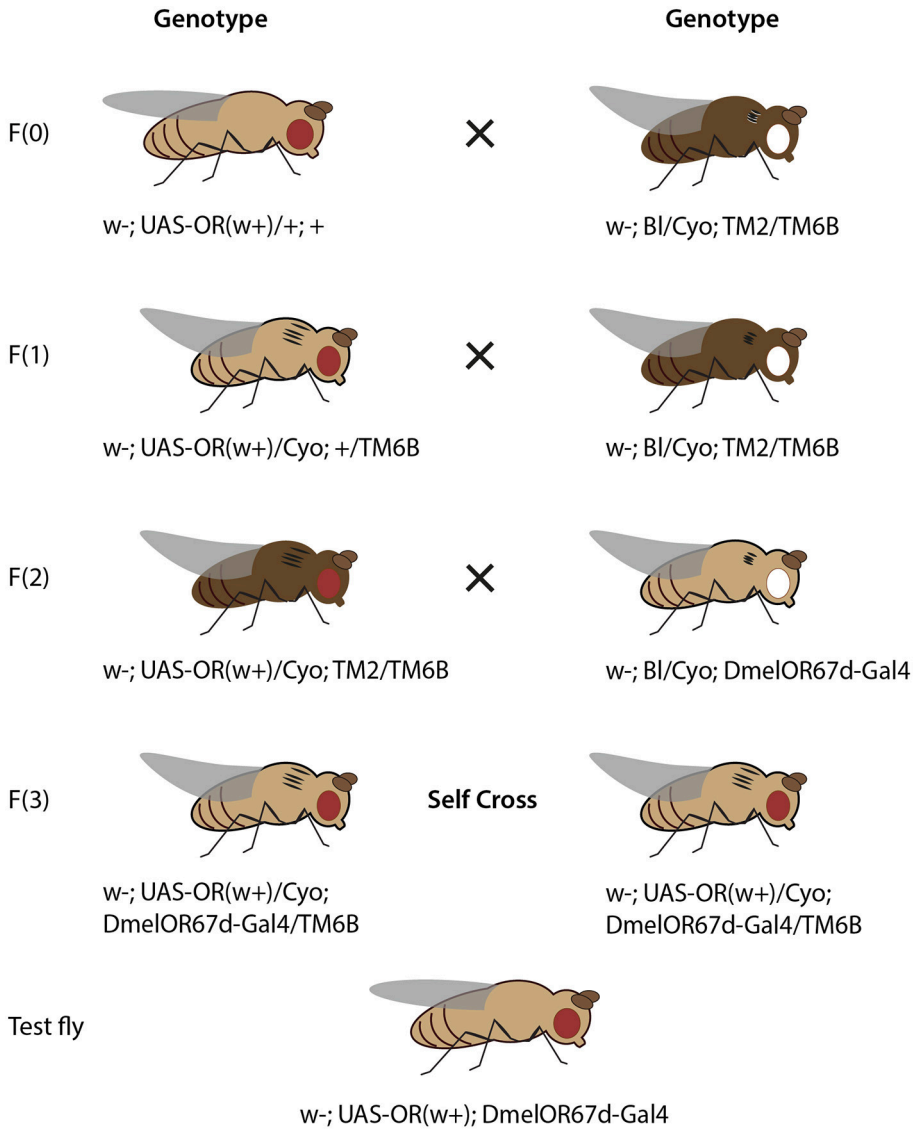

FIGURE 3 | Crossing scheme for heterologous expression of an OR transgene in OSNs in T1 sensilla, using DmelOR67d-Gal4 knock-in with knock-out of DmelOR67d coding sequence. Fly drawing by Katarina Eriksson (www.markadesign.se).

ORs in Drosophila OSNs) is shown in **Figures 1–3**. A best-case scenario time plan for the procedures described in the following section is shown in **Supplemental Figure 1**.

Molecular Cloning of Insect ORs

RNA Extraction and Purification

Dissect antennal (or other target) tissue from a sufficient number of insects into an empty 1.5-mL microcentrifuge tube held in liquid nitrogen, dry ice, or else standard ice. For *D. melanogaster,* 100 insects are recommended; for moths, 30 insects may be sufficient. Size of the antenna determines the number of specimen required.

Store target tissue in −80°C freezer, or proceed immediately to RNA extraction and purification. Follow standard protocol provided with extraction/purification kit/reagents.

Measure RNA quantity with photospectrometer or equivalent device and store RNA at −80°C or proceed immediately to the next step.

First-Strand cDNA Synthesis

Follow manufacturer's protocol for cDNA synthesis, with maximum quantity of RNA allowed within the volumetric parameters of the enzymatic reaction.

cDNA may commonly be diluted with ultra-pure water (e.g., at 1:1 ratio with cDNA sample) for PCR amplification assays, if necessary. However, dilution of cDNA may not be desirable, when the target genes show relatively low expression patterns compared to other ORs.

Store cDNA at −20°C or proceed immediately to the next step.

PCR Amplification of Target OR Sequence

Generate gene specific primers (GSPs) for PCR amplification of the entire open reading frame (ORF) of the target OR. Utilize forward primers (GSP1) that begin with the start codon and reverse primers (GSP2) that begin with the reverse-complement of the stop codon. If the start to stop codon primers are not ideal for PCR amplification due to mismatched melting temperatures (Tm, greater than 5°C difference) or other factors, it is advisable to design primers upstream or downstream of the ORF, respectively. If positive control primers are not previously available, the Orco gene could serve as a target to control for gene amplification in antennal tissue, since it is always expressed together with ORs and displays high expression in antennal tissue.

Conduct PCR amplification reaction with a DNA polymerase system that includes 3′ to 5′ exonuclease (proofreading) function. At this step, use of a proofreading *Taq* polymerase is critical; it drastically reduces the likelihood of obtaining unusable plasmid clones that contain OR inserts with incorrect sequence. Set up one PCR reaction per target OR, with positive (e.g., Orco) and negative (e.g., no template) control, according to manufacturers protocol. Run PCR amplification reaction in thermocycler machine according to manufacturers specifications for the *Taq* polymerase system, with annealing temperature 3°C less than primer melting temperature (Tm) and 30–35 amplification cycles (standard running time, ca. 2 h).

Store PCR overnight at 4°C, for longer periods at −20°C, or proceed immediately to next step.

Gel Analysis of PCR Product and Gel Purification

Run PCR products through 1.5% agarose gel for simultaneous verification of amplification and excision of OR-specific amplicon for purification. Expected band size for ORs is typically around 1200 base-pairs, as compared to fragments of standard DNA ladder.

Use low-intensity UV wavelength so as not to damage/mutate DNA, and minimize exposure time while cutting out the agarose gel that contains OR-specific fragments. Place excised gel in 1.5-mL microcentrifuge tubes and measure the mass of the added gel material. Gel may be frozen at −20°C for later use, or used immediately for the next step.

Purify OR-specific DNA from the gel with standard gel purification/extraction kit according to manufacturers protocol. Elute DNA in ultra pure sterile water or buffer provided with the kit.

Run a small aliquot (e.g., 5 μL) of purified DNA on a 1.5% agarose gel in order to verify success of the procedure and ensure the presence of only OR-specific DNA at the expected size.

Store gel-purified OR DNA at −20°C or proceed immediately to next step.

TOPO/Gateway Cloning of Target OR Sequence and Construct Confirmation

While the use of *Taq* polymerase with proofreading function is essential to ensure accurate amplification of the target sequence, it results in the removal of adenosine overhang nucleotides at the 5′ and 3′ ends of the DNA amplicon, which is a feature of standard *Taq* polymerase. These adenosine nucleotides are critical for the function of the TOPO cloning system. Thus, it is necessary, after gel purification, to enzymatically add the adenosine overhangs to the target OR sequence to be cloned.

Use 10 μL of gel-purified DNA, 1.2 μL of 10× PCR buffer, 1 μL of 10 mM dNTPs (both included in TOPO cloning kit), and 0.5 μL of standard *Taq* polymerase (without proofreading activity, not included in TOPO cloning kit). It is critical to use only the buffer supplied with the TOPO cloning kit; this buffer is compatible with downstream cloning steps. Mix contents and incubate at 72°C for 10 min. Proceed immediately to next step.

Add 4 μL of previous reaction, 1 μL of salt solution (provided with TOPO cloning kit) and 1 μL of topoisomerase vector mix (provided with TOPO cloning kit). Mix and incubate at room temperature (22–23°C) for more than 5 min, but less than 30 min. For inserts larger than 1 kb in size, the longer incubation time is recommended.

Toward the end of the incubation period, thaw appropriate number of aliquots of One Shot Competent *E. coli* (provided with TOPO cloning kit) on ice. Mix 2 μL of previous reaction with *E. coli* and chill on ice for greater than 5 min, but less than 30 min.

Heat shock cell/plasmid mixture at 42°C for 30 s, and place tube promptly on ice. Add 250 μL of SOC media (provided along with *E. coli* tubes) to cells and grow at 37°C for at least 1 h in incubator shaker. Apply entire contents of cell culture on prepared LB+Spectinomycin (50 μg/ml) bacterial growth plates and incubate overnight at 37°C. Plates may be stored at 4°C for up to 1 month.

To ensure appropriate expression of the OR transgene in *D. melanogaster*, orientation of the insert from 5′ to 3′ with reference to the attL1 element in the TOPO plasmid is required. To verify correct orientation of the insert, a standard colony PCR protocol is followed, with amplification using one GSP and one TOPO plasmid primer (GW1 or GW2); either combination of GW1 and GSP2 or GW2 and GSP1 will suffice. For either of these combinations, amplification of a PCR product (ca. 1.3 kb) will only occur if the insert is positioned in the plasmid in the desired orientation.

Typically, screening of 4–8 colonies with this assay is sufficient to identify a clone with the insert in the desired orientation. First, select colonies and transfer them each to a 1.5-mL microcentrifuge tube with 50 μL of LB plus spectinomycin (50 μg/ml) growth medium. Incubate culture at 37°C in incubator shaker for at least 1 h. In the meantime, prepare PCR reactions with master mix appropriate to the number of colonies being assayed. Using a *Taq* polymerase system, without proofreading function, a standard PCR reaction shall be prepared with 2 μL of each colony culture to be added to each PCR reaction tube. Remainder of colony culture is to be stored at 4°C for later use. For the amplification procedure, standard thermocycling parameters shall be followed according to the *Taq* polymerase system being used, with a 5 min extension period per cycle, and 30–35 amplification cycles. Ensuing gel analysis of PCR amplification products on a 1.5% agarose gel will confirm the presence of amplicon, and thus correct orientation of the insert.

For each TOPO/OR construct, one or more colony cultures with insert may be selected for further processing. After PCR assay and confirmation, the remainder of the colony culture is added to a culture tube with 3 mL of LB plus spectinomycin (50 μg/ml), and this culture is grown overnight at 37°C in a shaker incubator. After overnight growth, the culture may be stored at 4°C for 2–3 weeks or used immediately in the next step.

Using a standard plasmid mini prep purification kit, the culture is to be processed according to manufacturers protocol. Elute plasmid DNA in ultra-pure sterile water or supplied elution buffer and measure concentration of plasmid preparation with photospectrometer or equivalent equipment.

Confirm the sequence of the insert via sequencing reactions with GW1 and GW2 primers supplied with the TOPO cloning kit. This step is critical. Attempts to generate transgenic fly lines without verifying sequence beforehand may lead to otherwise avoidable failure of the experiment.

Store plasmid at −20°C until completion of the sequencing reactions. Discard all plasmids with incorrect sequence or errors otherwise. Select one plasmid with correct sequence for further processing.

Clonase Transfer of OR Insert to Gateway Destination Vector

Using the TOPO/OR and pUASg.attB plasmids diluted to specified concentrations, mix 6 μL of TOPO/OR, 2 μL of pUASg.attB, and 2 μL of the LR clonase enzyme (Thermo Fisher Scientific, USA) and incubate at 25°C for 1 h.

Add 1 μL of proteinase K (supplied with LR Clonase kit) to terminate previous reaction. Mix and incubate at 37°C for 10 min. This step is critical. If omitted, downstream outcomes will not be successful.

During the incubation period, thaw appropriate number of aliquots of One Shot Competent *E. coli* (provided with TOPO cloning kit) on ice. Mix 2.5 μL of the clonase reaction with *E. coli* and chill on ice for greater than 5 min, but less than 30 min.

Heat shock cell/plasmid mixture at 42°C for 30 s, and place tubes promptly on ice. Add 250 μL of SOC media (provided with *E. coli* tubes) to cells and grow at 37°C for at least 1 h in incubator shaker. Apply entire contents of cell culture on previously prepared LB+Ampicilin (50 μg/ml) bacterial growth plates and incubate overnight at 37°C. Plates may be stored at 4°C for up to 1 month.

On account of positive selection of pUASg.attB with OR insert, and negative selection against bacteria with TOPO/OR plasmid (these contain Spectinomycin but not Ampicillin resistance genes) and also those with pUASg.attB lacking OR insert (these contain lethal gene whose gene product results in death of One Shot *E. coli)*, all bacterial colonies on the growth plate will contain the pUASg.attB with OR insert in the correct orientation. Thus, colony PCR is not necessary at this step to confirm presence and orientation of the insert.

For each pUASg.attB/OR construct, transfer one colony to a culture tube with 3 mL of LB plus ampicillin (50 μg/ml), and grow the culture overnight at 37°C in shaker incubator. After overnight growth, culture may be stored at 4°C for up to 2–3 weeks or used immediately in the next step.

Using a standard plasmid mini- or midi-prep purification kit, the culture is to be processed according to manufacturers protocol. Elute plasmid DNA in ultra-pure sterile water and measure concentration of plasmid preparation with photospectrometer or equivalent equipment.

Confirm the sequence of the insert via sequencing reactions with UAS1 and UAS2 sequencing primers (described in **Table 1**). This step is critical. Attempts to generate transgenic fly lines without verifying sequence beforehand may lead to otherwise avoidable failure of the experiment.

Store plasmid at −20°C until completion of the sequencing reactions. Discard all plasmids with incorrect sequence or errors otherwise. Select one plasmid with correct sequence for injection in fly embryos.

For expression in the ab3A empty neuron system, it is desirable to insert the UAS-OR construct on the 3rd chromosome. Therefore, it is recommended that injections are made into embryos of the following genetic background:

y w M{eGFP.vas-int.Dm}ZH-2A; +; M{RFP.attP}ZH-86Fb; +

For expression in the T1 neuron system, it is desirable that the UAS-OR construct is inserted on the 2nd chromosome. Therefore, it is recommended that injections are made into embryos of the following genetic background:

y w M{eGFP.vas-int.Dm}ZH-2A; M{RFP.attP}ZH-51D; +; +

Injections are outsourced to a company providing *Drosophila* embryo injection services. Indeed, fly strains exist that contain landing sites at different locations on the second and third chromosomes. The recommended strains have been selected due to current availability as well as relatively high genomic integration efficiency and transgene expression. Consultation with fly embryo injection companies are advised to determine the best solutions with respect to available fly strains for this purpose.

Transgenic Expression of ORs in Drosophila OSNs

In order to express the OR transgene (UAS-ORx) in OSNs of either ab3 or T1 sensilla, it is necessary to push the transgene through a series of genetic crosses (**Figures 2**, **3**). Injections are made into a fly strain with white-eye mutation (w−) and the UAS-OR construct carries a rescue gene for the white-eye phenotype. Therefore, transgenic flies obtained after injections will have orange/red eyes and a genotype, w−; +; UAS-ORx (w+)/+, for use in ab3 system, or alternatively w−; UAS-ORx(w+)/+, +, for use in T1 system. A series of initial crosses are necessary to screen for the presence of transgene. While it is possible for the end-user to obtain larvae directly from injected embryos and screen for transgenic strains in the laboratory, this is labor intensive and not recommended. Alternatively, these steps are typically offered as service by fly-injection companies for a small fee above and beyond baseline injection costs. For further details on balancer chromosome phenotypes see Greenspan (1997). All stock flylines used for crosses mentioned below are available upon request from our laboratory.

Fly Crossing Scheme for the ab3A Empty Neuron System

The OR transgene must be crossed into the ΔHalo genetic background, which contains a chromosomal deletion spanning the location that includes the OR22a/b locus (Dobritsa et al., 2003; Gross et al., 2003). An outline of the required crosses is shown in **Figure 2**.

Cross 1. Cross w; +; UAS-ORx(w+)/+ to the double balancer strain, w; Bl/Cyo; TM2/TM6b. Select progeny with red eyes (w+), curly wings (Cyo) and tubby phenotype (with a cluster of bristles on the humerus, TM6b): w; +/Cyo; UAS-ORx(w+)/TM6b. The *ebony* phenotype features darker pigmentation and presents in flies with both third chromosome balancers (TM2/TM6B). In this schema the OR transgene is present on the third chromosome, its selection is thus mutually exclusive with the ebony phenotype.

Cross 2. Cross selected progeny again to w; Bl/Cyo; TM2/TM6b. Select progeny with red eyes, curly wings, tubby phenotype and short bristles (Bl), with genotype: w−; Bl/Cyo; UAS-ORx(w+)/TM6b. Since the ΔHalo mutation has no phenotypic markers, and is introduced in a genetic background with wild-type (longer) bristles, it is necessary to first pass the OR transgene through a short bristle phenotype in order to be able to discriminate the ΔHalo chromosome from its counterpart wild-type chromosome present in the original transgenic flies received.

Cross 3. Cross selected progeny to w−; ΔHalo/Cyo; TM2/TM6b. Select progeny with red eyes, curly wings, tubby and wild-type bristles (ΔHalo), with genotype, w−; ΔHalo/Cyo; UAS-ORx(w+)/TM6b.

Cross 4. Self-cross selected male and female progeny. Select and breed male and female progeny with red eyes, curly wings, and wild type bristles, without tubby, w−; ΔHalo/Cyo; UAS-ORx, in order to establish a stable stock of fly lines that are ready for the experimental cross and downstream electrophysiological assay. In this stock line, the ΔHalo chromosome is maintained in the presence of the Cyo balancer. While ΔHalo homozygous flies are viable and obtained for downstream assay, they are not fit for reproduction and are relatively sick. It is thus advisable to also maintain a stock of flies with genotype, w−; Bl/Cyo; UAS-ORx(w+)/TM6b, obtained after Cross 2 (above).

Expression Cross. Cross w−; ΔHalo/Cyo; UAS-ORx(w+) to w; ΔHalo/Cyo; DmelOR22a-Gal4(w+). Select female progeny with red eyes and straight wings, w−; ΔHalo/ΔHalo; DmelOR22a-Gal4(w+)/UAS-ORx(w+). These flies are to be used for physiological assay, as described below. Since both the Gal4 and UAS constructs in this system are maintained on the third chromosome, it is not possible to maintain a stable stock of these flies for physiological assays on demand. The expression cross must be made as described above whenever OR assays in the ab3A empty neuron system is required.

Fly Crossing Scheme for the T1 Knock-In Neuron System

The OR transgene must be crossed into the OR67d-knockout/Gal4-knock-in genetic background, which contains a Gal4 transgene in place of the native OR67d gene, and under control of the native OR67d promoter (Kurtovic et al., 2007). An outline of the required crosses is shown in **Figure 3**.

Cross 1. Cross w; UAS-ORx(w+)/+; + to the double balancer strain, w; Bl/Cyo; TM2/TM6b. Select progeny with red eyes (w+), curly wings (Cyo) and tubby phenotype (TM6b), with genotype: w−; UAS-ORx(w+)/Cyo; +/TM6b.

Cross 2. Cross selected progeny again to w; Bl/Cyo; TM2/TM6b. Select progeny with red eyes, curly wings, tubby phenotype and ebony body color, with genotype: w−; UAS-ORx(w+)/Cyo; TM2/TM6b.

Cross 3. Cross selected progeny to w−; Bl/Cyo; OR67d-Gal4. Select progeny with red eyes, curly wings, wild-type bristles, and tubby phenotype, with genotype w−; UAS-ORx(w+)/Cyo; OR67d-Gal4/TM6b.

Cross 4. Self-cross selected male and female progeny. Select and breed male and female progeny with red eyes, straight wings, and without tubby phenotype, with genotype: w−; UAS-ORx(w+); OR67d-Gal4. In this case, these flies are viable for stock breeding and are also of the correct genotype for experimental testing.

Single Sensillum Recordings

Mounting

Trap a fly inside a 200-μl pipette tip. Horizontally cut the pipette tip close to the head and push the head slightly out of the pipette tip. Place the pipette tip containing the fly facing upwards on dental wax on a microscope slide. Push the antennae on the glass slide fixed with dental wax on the full-length microscope slide.

Fix the glass capillary on dental wax on the microscope slide. Use the glass capillary to push the antenna down by pressing the section between the second and third antennal segment. In the case of transgene ORs expressed in the ab3A OSN, manipulate the glass capillary until exposing the dorso-medial area of the antenna. A cluster of thumb-shaped sensilla (large basiconic sensilla) facing upwards should be visible. For testing transgene ORs that are expressed in trichoid T1 sensilla, manipulate the glass capillary to firmly press the lateral side of the antenna against the microscope slide. The corresponding long pointy sensilla (T1) are then located at the superior side of the antenna from the lateral view. Anatomical maps of the *D. melanogaster* antenna and sensillum types are found in de Bruyne et al. (2001), Stocker (2001), Dobritsa et al. (2003), and Couto et al. (2005). Guidance to perform electrophysiological recordings can be found in Pellegrino et al. (2010) and in Benton and Dahanukar (2011).

Recording

Place the mounted fly under the microscope and penetrate its right eye with the tungsten reference electrode. At high magnification of the microscope, use the micromanipulator to move the tungsten recording electrode along the antenna.

Penetrate either large basiconic sensilla located in the dorso-medial area of the antennae (ab3, empty neuron system), or long trichoid sensilla at the tip of the antennae (T1 knock-in neuron system).

Gently manipulate the recording electrode along the base of the sensilla until a clear pattern of neuronal activity is established (monitored by Autospike; Syntech, Kirchzarten).

Identification of Sensillum Identity

After making contact, confirm sensillum identity prior to testing.

AB3 sensillum. Stable recordings from ab3 sensilla (Dobritsa et al., 2003) will produce spike trains from two OSNs, ab3A and ab3B, with two distinct amplitudes (**Figure 4A**). A response to stimulation with 2-heptanone, targeting the native Or85b expressed in ab3B, serves as double control: it confirms proper sensillum contact and the identity of the ab3 sensillum.

Regular spontaneous activity of the second neuron ab3A is indicative of a functional transgenic OR. The frequency of spontaneous neuronal activity of OSNs has been shown to be determined by the specific receptor protein that is expressed in the neuron (Hallem et al., 2004), variance of this feature is thus to be expected. However, response to stimulation with the wild type ligands, ethyl hexanoate or ethyl butyrate, is absent or modified and confirms that OR22a is not expressed (**Figure 4B**).

Last but not least, contact with the wrong large basiconic sensillum subtype (ab1 or ab2) can be ruled out by testing their natural ligands. The ab1 sensillum contains four OSNs (with varying spike amplitudes), one of which is responsive to CO_2: breathing gently over the fly will produce a response. For ab2, containing two OSNs, a ethyl-3-hydroxybutyrate stimulus produces a strong response.

T1 sensillum. When recording from T1 sensilla (Kurtovic et al., 2007), only one OSN responds (**Figure 4C**). The two other types of trichoid sensilla, T2 and T3, contain 2 and 3 neurons, respectively, facilitating discrimination between different trichoid sensilla. The OR insert in T1 is confirmed through lack of a response to cVA (**Figure 4D**).

Odor Stimulation

Apply 10 μl of test chemical solvent dilutions to filter paper discs inside Pasteur pipettes. Pulses of charcoal-filtered air (2.5 ml) through the pipette are delivered by a stimulus controller (Syntech, Kirchzarten, Germany), lasting at least 0.5 s, into glass tubing delivering air to the fly. Verify sensillum identity before testing. Once contact with correct sensillum subtype is established, sequentially deliver the test panel of compounds. Response magnitude is determined by counting the number of spikes before and after the onset of a response.

Alternatively, test stimuli can be provided by the effluent of a gas chromatograph (GC-SSR). The main advantages of using the GC for stimulation are discussed below (Section Testing Odourants).

ANTICIPATED RESULTS AND TROUBLESHOOTING

Molecular Cloning and Heterologous Expression

Most attempts to amplify the ORF of a determined OR and clone it into the TOPO entry vector will be successful with little difficulty. Common problems may be remedied after consultation with the troubleshooting section of the TOPO cloning user manual. It must be noted, however, that in some cases, attempts to amplify or obtain clones with the OR construct in the correct orientation can be unsuccessful. In case of amplification issues, it may be necessary to optimize the PCR amplification with gradient PCR or selection of new primer pairs that are more compatible with each other and the target cDNA in question.

Pertaining to issues with identifying TOPO clones with the desired insert in the correct orientation, it may be necessary, during the colony PCR screening step, to assay both combination of plasmid/insert primer pairs, due to primer incompatibility issues. Otherwise, various unknown and unapparent factors may render some OR constructs refractory to plasmid vector propagation. In our experience, this is rare, but may happen; with patience and effort these molecular obstacles can usually be overcome.

In test flies, OR transgenes are expressed in either ab3A or T1 OSNs, which lack their native OR22a/b or OR67d receptors, respectively. This should be verified though PCR assay of transgene OR expression in antennae of progeny obtained through experimental crosses. This can be done by following the procedures described in section 3.1.1 to 3.1.4, using the antennae of 100 test flies as starting material. It is our experience that most ORs will be expressed appropriately in the *D. melanogaster* antennae, once the appropriate fly crosses have been made. However, in few cases, ORs are refractory to expression in these sensilla, for yet unknown reasons.

Response of OSNs Expressing Novel ORs

The functionality of heterologous expression of ORs in ab3A and T1 OSNs is assessed by SSR. As mentioned above, the ab3 basiconic sensilla house two neurons, ab3A, which natively expresses OR22a (tuned to ethyl hexanoate and ethyl butyrate) and ab3B, which expresses OR85b (tuned to 2-heptanone). Identity and functionality of this sensillum can be verified through stimulation of the ab3B neuron with 2-heptanone.

If ab3A sensilla, expressing a novel OR, should respond to ethyl hexanoate or ethyl butyrate, further testing with other OR22a ligands such as methyl hexanoate, isobutyl acetate and methyl octanoate (Hallem and Carlson, 2006) can help to determine whether the native DmelOR22a or the experimental transgenic OR produce this response. Expression of the transgene OR and lack of expression of the native OR22a receptor can also be confirmed with a PCR assay, as described above. If DmelOR22a is indeed present, it is likely due to erroneous fly-crossings that failed to exclude the wild-type second chromosome. In this case, it will be necessary to carefully perform the fly-crossing schema again to ensure that the ΔHalo

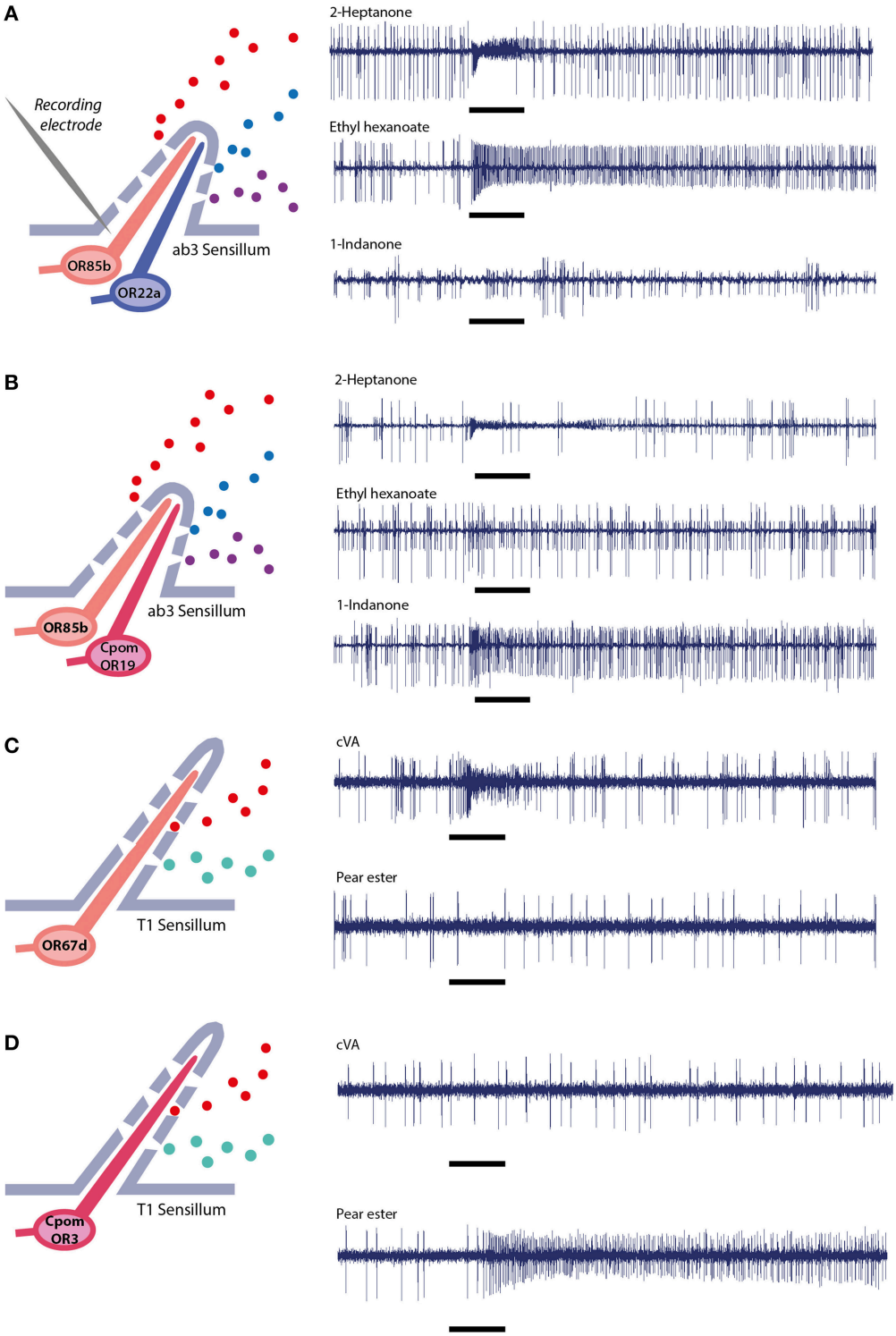

FIGURE 4 | Single sensillum electrophysiological recordings. In ab3 empty neuron system, **(A)** wild-type flies expressing native ORs, **(B)** mutant flies expressing native OR85b in the small neuron and transgenic CpomOR19 from *C. pomonella* in the large neuron (Gonzalez et al., 2015). In T1 empty neuron system, **(C)** wild-type flies expressing native OR67d, **(D)** mutant flies expressing transgenic CpomOR3 (Bengtsson et al., 2014).

chromosome is present in place of a wild-type chromosome containing DmelOR22a.

On the other hand, even if receptors are functionally expressed and confer a background-firing rate on respective ab3A and T1 OSNs, test odourants may not elicit significant responses. The solution is to use a broader test panel of odourants, taking into account a diversity of ecological sources of odourants that are representative of the olfactory environment of the insect being studied. Using volatile collections from natural substrates in combination with GC-SSR is an option.

In a functional transgenic ab3A system, only ligands activating the transgenic OR will produce a response from ab3A neurons. Recently, we have deorphanized CpomOR19 and SlitOR19, of *C. pomonella* and *Spodoptera littoralis*, using the ab3A system. A response to 1-indanone was recorded only after expression of CpomOR19 or SlitOR19 in ab3A OSNs, and not from wild-type *D. melanogaster* (**Figures 4A,B**; Gonzalez et al., 2015).

Expression of transgenic candidate PRs or other ORs in T1 neurons is characterized by an irregular firing rate (Ronderos et al., 2014). Wild-type flies will show an intense and long-lasting response when stimulated with cVA, while experimental flies will respond with a less intense but more irregular pattern to the ligands of the respective transgene ORs (**Figures 4C,D**).

CpomOR3 belongs phylogenetically to the clade of moth pheromone receptors and is tuned to the plant volatile pear ester. After functional expression of CpomOR3 in neurons of either ab3 or T1 sensilla, responsiveness and tuning were equally specific and sensitive (Bengtsson et al., 2014).

Sensillum Environment and OR Function

Systematic investigations of the OR repertoires of *Drosophila* and the malaria mosquito *Anopheles gambiae* demonstrate that the ab3A empty neuron is a faithful expression system for insect OR genes. OR response profiles in native neurons and in the empty neuron, generally resemble each other, but are not identical (Dobritsa et al., 2003; Hallem et al., 2004; Carey et al., 2010). However, not all receptors will work in ab3A neurons. For example, only 50 out of 72 cloned *A. gambiae* ORs were functional in the empty neuron (Carey et al., 2010). A similar percentage of *D. melanogaster* ORs were also not functional in the ab3A empty neuron (Hallem et al., 2004). In cases where transgene ORs are expressed but not functional in ab3A neuron, the background neuronal firing rate phenocopies the ab3A empty neuron condition with regular cluster bursts of multiple action potentials (Dobritsa et al., 2003).

Advances in transcriptomics and the molecular basis of odourant reception in insects will help us to understand what facilitates or impedes correct function of ORs. One explanation is that the cellular environment contributes membrane-bound proteins, such as sensory neuron membrane proteins (SNMPs) and extracellular odourant- or pheromone-binding proteins (OBPs, PBPs), which are known to mediate interactions between ORs or PRs and odourant molecules (Nichols and Vogt, 2008; Leal, 2013; Li et al., 2014; Vogt et al., 2015). Expression patterns of SNMPs and OBPs have been investigated across olfactory organs (Vogt et al., 2002; Shanbhag et al., 2005; Benton et al., 2007), however detailed expression patterns of these genes at the cellular level, with reference to ORs, remain to be fully described.

The combined role of ORs and OBPs, and PRs and PBPs, respectively, in odourant detection and discrimination, has been confirmed by co-expression analysis and by heterologous expression in *Xenopus* (Schultze et al., 2013; Chang et al., 2015). This is in line with the observation that some ORs are functional only in trichoid sensilla. Presence of the extracellular protein LUSH is necessary for pheromone-sensitive OSNs in *Drosophila* T1 sensilla (Xu et al., 2005; Laughlin et al., 2008). Similarly, DmelOr83c does not produce a response at all when transgenically expressed in basiconic sensilla OSNs, but requires factors present in trichoid sensilla, including SMNP1 (Ronderos et al., 2014).

Testing Odourants

Odourants used for functional characterization of insect ORs comprise a range of compounds which greatly differ in molecular weight and, accordingly, also in vapor pressure and evaporation rates. In addition, when compounds are formulated onto passive dispensers, their physicochemical affinity to the substrate, including polarity, will modify evaporation rates. Release rates of the odourants included in a test panel will often differ by several orders of magnitude. Yet, these differences in release rates are notoriously ignored or underestimated. For valid structure-activity comparisons, the amounts of test compounds delivered to the antenna need to be corrected for differences in evaporation rates (Bengtsson et al., 1990).

Chemical impurities of test odourants are another serious error source. Standards of natural or synthetic compounds invariably contain impurities, which may be more active than the test compound itself. Even impurities present in trace amounts may elicit strong responses, since ORs are indeed known to be strongly tuned to their key ligands. Last but not least, availability of synthetic standards is often a limiting factor.

Using GC-SSR for stimulation elegantly accounts for these main concerns: release rates, chemical purity and availability of standards. Headspace collections from biological substrates, for example, leaves or fruits of higher plants, will typically contain several dozens of volatiles. Through the GC column, these compounds are delivered at known amounts, independently of vapor pressure. Choice of the column and temperature programme will ensure delivery of pure compound at baseline separation. This includes even geometrical or optical isomers of plant volatiles, which rarely are available as pure standards.

CONCLUSION

We expect heterologous expression of insect ORs in *Drosophila* OSNs to make a significant future contribution to the identification of insect semiochemicals, and to investigations of the phylogenetic progression and the functional properties of ORs.

AUTHOR CONTRIBUTIONS

PW: conceived the idea of the manuscript. FG, WW, and PW designed and prepared the figures. FG, WW, and PW wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2016.00024

Supplemental Figure 1 | Timeframe for molecular cloning and generation of a transgenic fly line. Multiple ORs can be processed in parallel. Time for molecular work is a best-case scenario, assuming access to DNA sequencing of the generated plasmids. Timeline for obtaining transgenic flies after sending transformation/expression plasmids depends on micro-injection company.

REFERENCES

Anderson, A. R., Wanner, K. W., Trowell, S. C., Warr, C. G., Jaquin-Joly, E., Zagatti, P., et al. (2009). Molecular basis of female-specific odorant responses in *Bombyx mori*. *Insect Biochem. Mol. Biol.* 39, 189–197. doi: 10.1016/j.ibmb.2008.11.002

Ansebo, L., Coracini, M. D. A., Bengtsson, M., Liblikas, I., Ramírez, M., Borg-Karlsson, A.-K., et al. (2004). Antennal and behavioural response of codling moth *Cydia pomonella* to plant volatiles. *J. Appl. Entomol.* 128, 488–493. doi: 10.1111/j.1439-0418.2004.00878.488–493

Arn, H., Städler, E., and Rauscher, S. (1975). The electroantennographic detector - a selective and sensitive tool in the gas chromatographic analysis of insect pheromones. *Z. Naturforsch.* 30c, 722–725. doi: 10.1515/znc-1975-11-1204

Arn, H., Tóth, M., and Priesner, E. (1992). *List of Sex Pheromones of Lepidoptera and Related Attractants*, *2nd Edn*. Montfavet: International Organization for Biological Control.

Bäckman, A.-C., Bengtsson, M., Borg-Karlsson, A.-K., Liblikas, I., and Witzgall, P. (2001). Volatiles from apple eliciting antennal responses in female codling moth *Cydia pomonella* (L.) (Lepidoptera: Tortricidae): effect of plant injury and sampling technique. *Z. Naturforsch.* 56c, 262–268. doi: 10.1515/znc-2001-3-415

Bengtsson, J. M., Gonzalez, F., Cattaneo, A. M., Montagné, N., Walker, W. B., Bengtsson, M., et al. (2014). A predicted sex pheromone receptor of codling moth *Cydia pomonella* detects the plant volatile pear ester. *Front. Ecol. Evol.* 2:33. doi: 10.3389/fevo.2014.00033

Bengtsson, J. M., Trona, F., Montagné, N., Anfora, G., Ignell, R., Witzgall, P., et al. (2012). Putative chemosensory receptors of the codling moth, *Cydia pomonella,* identified by antennal transcriptome analysis. *PLoS ONE* 7:e31620. doi: 10.1371/journal.pone.0031620

Bengtsson, M., Liljefors, T., Hansson, B. S., Löfstedt, C., and Copaja, S. V. (1990). Structure-activity relationships for chain-shortened analogs of (Z)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*. *J. Chem. Ecol.* 16, 667–684. doi: 10.1007/BF01016478

Benton, R., and Dahanukar, A. (2011). "Electrophysiological recording from *Drosophila* taste sensilla," in *Drosophila Neurobiology: A Laboratory Manual*, eds B. Zhang, M. R. Freeman, and S. Waddell (New York, NY: Cold Spring Harbor Protocols), 839–850.

Benton, R., Vannice, K. S., and Vosshall, L. B. (2007). An essential role for a CD36-related receptor in pheromone detection in *Drosophila*. *Nature* 450, 289–293. doi: 10.1038/nature06328

Bischof, J., Maeda, R. K., Hediger, M., Karch, F., and Basler, K. (2007). An optimized transgenesis system for *Drosophila* using germ-line-specific ϕC31 integrases. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3312–3317. doi: 10.1073/pnas.0611511104

Brand, A. H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118, 401–415.

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. doi: 10.1038/nature08834

Chang, H., Liu, Y., Yang, T., Pelosi, P., Dong, S., and Wang, G. (2015). Pheromone binding proteins enhance the sensitivity of olfactory receptors to sex pheromones in *Chilo suppressalis*. *Sci. Rep*. 5:13093. doi: 10.1038/srep13093

Clyne, P. J., Warr, C. G., Freeman, M. R., Lessing, D., Kim, J., and Carlson, J. R. (1999). A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron* 22, 327–338. doi: 10.1016/S0896-6273(00)81093-4

Coracini, M., Bengtsson, M., Liblikas, I., and Witzgall, P. (2004). Attraction of codling moth males to apple volatiles. *Ent. Exp. Appl.* 110, 1–10. doi: 10.1111/j.0013-8703.2004.00124

Corcoran, J. A., Jordan, M. D., Carraher, C., and Newcomb, R. D. (2014). A novel method to study insect olfactory receptor function using HEK293 cells. *Insect Biochem. Mol. Biol.* 54, 22–32. doi: 10.1016/j.ibmb.2014.08.005

Couto, A., Alenius, M., and Dickson, B. J. (2005). Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Curr. Biol.* 15, 1535–1547. doi: 10.1016/j.cub.2005.07.034

de Bruyne, M., Foster, K., and Carlson, J. R. (2001). Odor coding in the *Drosophila* antenna. *Neuron* 30, 537–552. doi: 10.1016/S0896-6273(01)00289-6

Dethier, V. G. (1947). *Chemical Insect Attractants and Repellents*. Philadelphia, PA: The Blakiston Company.

Dobritsa, A. A., van der Goes van Naters, W., Warr, C. G., Steinbrecht, R. A., and Carlson, J. R. (2003). Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron* 37, 827–841. doi: 10.1016/S0896-6273(03)00094-1

El-Sayed, A. M. (2015). *The Pherobase: Database of Insect Pheromones and Semiochemicals*. Available online at: http://www.pherobase.com

Gao, Q., and Chess, A. (1999). Identification of candidate *Drosophila* olfactory receptors from genomic DNA sequence. *Genomics* 60, 31–39. doi: 10.1006/geno.1999.5894

Goldman, A. L., van der Goes van Naters, W., Lessing, D., Warr, C. G., and Carlson, J. R. (2005). Coexpression of two functional odor receptors in one neuron. *Neuron* 45, 661–666. doi: 10.1016/j.neuron.2005.01.025

Gonzalez, F., Bengtsson, J. M., Walker, W. B., Rodrigues-Sousa, M. F., Cattaneo, A. M., Montagné, N., et al. (2015). A conserved odorant receptor detects the same 1-indanone analogs in a tortricid and a noctuid moth. *Front. Ecol. Evol.* 3:131. doi: 10.3389/fevo.2015.00131

Greenspan, R. J. (1997). *Fly Pushing: The Theory and Practice of Drosophila Genetics*. New York, NY: Cold Springs Harbor Laboratory Press.

Große-Wilde, E., Svatos, A., and Krieger, J. (2006). A pheromone-binding protein mediates the bombykol-induced activation of a pheromone receptor *in vitro*. *Chem. Senses* 31, 547–555. doi: 10.1093/chemse/bjj059

Gross, S. P., Guo, Y., Martinez, J. E., and Welte, M. A. (2003). A determinant for directionality of organelle transport in *Drosophila* embryos. *Curr. Biol.* 13, 1660–1668. doi: 10.1016/j.cub.2003.08.032

Hallem, E. A., and Carlson, J. R. (2006). Coding of odors by a receptor repertoire. *Cell* 125, 143–160. doi: 10.1016/j.cell.2006.01.050

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. doi: 10.1016/j.cell.2004.05.012

Jiang, X.-J., Guo, H., Di, C., Yu, S., Zhu, L., Huang, L.-Q., et al. (2014). Sequence similarity and functional comparisons of pheromone receptor orthologs in two closely related *Helicoverpa* species. *Insect Biochem. Mol. Biol.* 48, 63–74. doi: 10.1016/j.ibmb.2014.02.010

Jordan, M. D., Anderson, A., Begum, D., Carraher, C., Authier, A., Marshall, S. D., et al. (2009). Odorant receptors from the light brown apple moth (*Epiphyas postvittana*) recognize important volatile compounds produced by plants. *Chem. Senses* 34, 383–394. doi: 10.1093/chemse/bjp010

Jurenka, R. (2004). "Insect pheromone biosynthesis," in *The Chemistry of Pheromones and Other Semiochemicals I*, ed S. Schulz (Berlin; Heidelberg: Springer), 97–132. doi: 10.1007/b95450

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. doi: 10.1016/j.jnrumeth.2006.07.005

Kreher, S. A., Kwon, J. Y., and Carlson, J. R. (2005). The molecular basis of odor coding in the *Drosophila* larva. *Neuron* 46, 445–456. doi: 10.1016/j.neuron.2005.04.007

Kreher, S. A., Mathew, D., Kim, J., and Carlson, J. R. (2008). Translation of sensory input into behavioral output via an olfactory system. *Neuron* 59, 110–124. doi: 10.1016/j.neuron.2008.06.010

Kurtovic, A., Widmer, A., and Dickson, B. J. (2007). A single class of olfactory neurons mediates behavioural responses to a *Drosophila* sex pheromone. *Nature* 446, 542–546. doi: 10.1038/nature05672

Laughlin, J. D., Ha, T. S., Jones, D. N., and Smith, D. P. (2008). Activation of pheromone-sensitive neurons is mediated by conformational activation of pheromone-binding protein. *Cell* 133, 1255–1265. doi: 10.1016/j.cell.2008.04.046

Leal, W. S. (2013). Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. *Annu. Rev. Entomol.* 58, 373–391. doi: 10.1146/annurev-ento-120811-153635

Leary, G. P., Allen, J. E., Bunger, P. L., Luginbill, J. B., Linn, C. E., Macallister, I. E., et al. (2012). Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14081–14086. doi: 10.1073/pnas.1204661109

Li, Z., Ni, J. D., Huang, J., and Montell, C. (2014). Requirement for *Drosophila* SNMP1 for rapid activation and termination of pheromone-induced activity. *PLoS Genet.* 10:e1004600. doi: 10.1371/journal.pgen.1004600

Light, D. M., and Beck, J. J. (2012). Behavior of codling moth (Lepidoptera: Tortricidae) neonate larvae on surfaces treated with microencapsulated pear ester. *Environ. Entomol.* 41, 603–611. doi: 10.1603/EN11273

Light, D. M., and Knight, A. (2005). Specificity of codling moth (*Lepidoptera*: *Tortricidae*) for the host plant kairomone, ethyl (2E,4Z)-2,4-decadienoate: field bioassays with pome fruit volatiles, analogue, and isomeric compounds. *J. Agric. Food Chem.* 53, 4046–4053. doi: 10.1021/jf040431r

Light, D. M., Knight, A. L., Henrick, C. A., Rajapaska, D., Lingren, B., Dickens, J. C., et al. (2001). A pear-derived kairomone with pheromonal potency that attracts male and female codling moth, *Cydia pomonella* (L.). *Naturwiss* 88, 333–338. doi: 10.1007/s001140100243

Liu, C., Liu, Y., Walker, W. B., Dong, S., and Wang, G. (2013). Identification and functional characterization of sex pheromone receptors in beet armyworm *Spodoptera exigua* (Hübner). *Insect Biochem. Mol. Biol.* 43, 747–754. doi: 10.1016/j.ibjmb.2013.05.009

Matarazzo, V., Clot-Faybesse, O., Marcet, B., Guiraudie-Capraz, G., Atanasova, B., Devauchelle, G., et al. (2005). Functional characterization of two human olfactory receptors expressed in the baculovirus Sf9 insect cell system. *Chem. Senses* 30, 195–207. doi: 10.1093/chemse/bji015

Mathew, D., Martelli, C., Kelley-Swift, E., Brusalis, C., Gershow, M., Samuel, A. D., et al. (2013). Functional diversity among sensory receptors in a *Drosophila* olfactory circuit. *Proc. Natl. Acad. Sci. U.S.A.* 110, E2134–E2143. doi: 10.1073/pnas.1306976110

Mitsuno, H., Sakurai, T., Murai, M., Yasuda, T., Kugimiya, S., Ozawa, R., et al. (2008). Identification of receptors of main sex-pheromone components of three Lepidopteran species. *Eur. J. Neurosci.* 28, 893–902. doi: 10.1111/j.1460-9568.2008.06429.x

Montagné, N., Chertemps, T., Brigaud, I., François, A., François, M. C., De Fouchier, A., et al. (2012). Functional characterization of a sex pheromone receptor in the pest moth *Spodoptera littoralis* by heterologous expression in *Drosophila*. *Eur. J. Neurosci.* 36, 2588–2596. doi: 10.1111/j.1460-9568.2012.08183.x

Nichols, Z., and Vogt, R. G. (2008). The SNMP/CD36 gene family in Diptera, Hymenoptera and Coleoptera: *Drosophila melanogaster*, *D. pseudoobscura, Anopheles gambiae, Aedes aegypti, Apis mellifera, and Tribolium castaneum. Insect Biochem. Mol. Biol.* 38, 398–415. doi: 10.1016/j.ibmb.2007.11.0033

Pellegrino, M., Nakagawa, T., and Vosshall, L. B. (2010). Single sensillum recordings in the insects *Drosophila melanogaster* and *Anopheles gambiae*. *J. Vis. Exp*. 36:e1725. doi: 10.3791/1725

Ronderos, D. S., Lin, C. C., Potter, C. J., and Smith, D. P. (2014). Farnesol-detecting olfactory neurons in *Drosophila*. *J. Neurosci.* 34, 3959–3968. doi: 10.1523/JNEUROSCI.4582-13.2014

Sakurai, T., Nakagawa, T., Mitsuno, H., Mori, H., Endo, Y., Tanoue, S., et al. (2004). Identification and functional characterization of a sex pheromone receptor in the silkmoth *Bombyx mori*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16653–16658. doi: 10.1073/pnas.0407596101

Schneider, D. (1957). Elektrophysiologische untersuchungen von chemo- and mechanorezeptoren der Antenne des Seidenspinners *Bombyx mori* L. *Z. Vergl. Physiol.* 40, 8–41. doi: 10.1007/BF00298148

Schultze, A., Pregitzer, P., Walter, M. F., Woods, D. F., Marinotti, O., Breer, H., et al. (2013). The co-expression pattern of odorant binding proteins and olfactory receptors identify distinct trichoid sensilla on the antenna of the malaria mosquito *Anopheles gambiae*. *PLoS ONE* 8:e69412. doi: 10.1371/journal.pone.0069412

Shanbhag, S. R., Müller, B., and Steinbrecht, R. A. (1999). Atlas of olfactory organs of *Drosophila melanogaster*: 1. Types, external organization, innervation and distribution of olfactory sensilla. *Int. J. Insect Morphol. Embryol.* 28, 377–397. doi: 10.1016/S0020-7322(99)00039-2

Shanbhag, S. R., Smith, D. P., and Steinbrecht, R. A. (2005). Three odorant-binding proteins are co-expressed in sensilla trichodea of *Drosophila melanogaster*. *Arthropod Struct. Dev.* 34, 153–165. doi: 10.1016/j.asd.2005.01.003

Stocker, R. F. (2001). *Drosophila* as a focus in olfactory research: mapping of olfactory sensilla by fine structure, odor specificity, odorant receptor expression, and central connectivity. *Microsc. Res. Tech.* 55, 284–296. doi: 10.1002/jemt.1178

Syed, Z., Ishida, Y., Taylor, K., Kimbrell, D. A., and Leal, W. S. (2006). Pheromone reception in fruit flies expressing a moth's odorant receptor. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16538–16543. doi: 10.1073/pnas.0607874103

Syed, Z., Kopp, A., Kimbrell, D. A., and Leal, W. S. (2010). Bombykol receptors in the silkworm moth and the fruit fly. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9436–9439. doi: 10.1073/pnas.1003881107

Tegler, L. T., Corin, K., Hillger, J., Wassie, B., Yu, Y., and Zhang, S. (2015). Cell-free expression, purification, and ligand-binding analysis of *Drosophila melanogaster* olfactory receptors DmOR67a, DmOR85b and DmORCO. *Sci. Rep.* 5:7867. doi: 10.1038/srep07867

Trona, F., Anfora, G., Balkenius, A., Bengtsson, M., Tasin, M., Knight, A., et al. (2013). Neural coding merges sex and habitat chemosensory signals in an insect herbivore. *Proc. Biol Sci.* 280, 20130267. doi: 10.1098/rspb.2013.0267

Trona, F., Anfora, G., Bengtsson, M., Witzgall, P., and Ignell, R. (2010). Coding and interaction of sex pheromone and plant volatile signals in the antennal lobe of the codling moth *Cydia pomonella*. *J. Exp. Biol.* 213, 4291–4303. doi: 10.1242/jeb.047365

Vogt, R. G., Große-Wilde, E., and Zhou, J. J. (2015). The lepidoptera odorant binding protein gene family: gene gain and loss within the GOBP/PBP complex of moths and butterflies. *Insect Biochem. Mol. Biol.* 62,142–153. doi: 10.1016/j.ibmb.2015.03.003

Vogt, R. G., Rogers, M. E., Franco, M. D., and Sun, M. (2002). A comparative study of odorant binding protein genes: differential expression of the PBP1-GOBP2 gene cluster in *Manduca sexta* (Lepidoptera) and the organization of OBP genes in *Drosophila melanogaster* (Diptera). *J. Exp. Biol.* 205, 719–744.

Vosshall, L. B., Amrein, H., Morozov, P. S., Rzhetsky, A., and Axel, R. (1999). A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell* 96, 725–736. doi: 10.1016/S0092-8674(00)80582-6

Wanner, K. W., Nichols, A. S., Allen, J. E., Bunger, P. L., Garczynski, S. F., Linn, C. E., et al. (2010). Sex pheromone receptor specificity in the European corn borer moth, *Ostrinia nubilalis*. *PLoS ONE* 5:e8685. doi: 10.1371/journal.pone.0008685

Xu, P., Atkinson, R., Jones, D. N., and Smith, D. P. (2005). *Drosophila* OBP LUSH is required for activity of pheromone-sensitive neurons. *Neuron* 45, 193–200. doi: 10.1016/j.neuron.2004.12.031

Xu, W., Papanicolaou, A., Liu, N. Y., Dong, S. L., and Anderson, A. (2015). Chemosensory receptor genes in the Oriental tobacco budworm *Helicoverpa assulta*. *Insect Mol. Biol.* 24, 253–263. doi: 10.1111/imb.12153

Zhang, D. D., and Löfstedt, C. (2013). Functional evolution of a multigene family: orthologous and paralogous pheromone receptor genes in the turnip moth, *Agrotis segetum*. *PLoS ONE* 8:e77345. doi: 10.1371/journal.pone.0077345

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A Conserved Odorant Receptor Detects the Same 1-Indanone Analogs in a Tortricid and a Noctuid Moth

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Odorant receptors (ORs) interface animals with airborne chemical signals. They are under strong selection pressure and are therefore highly divergent in different taxa. Yet, some OR orthologs are highly conserved. These ORs may be tuned to odorants of broad importance, across species boundaries. Two widely distributed lepidopteran herbivores, codling moth *Cydia pomonella* (Tortricidae) feeding in apples and pears, and the African cotton leafworm *Spodoptera littoralis* (Noctuidae), a moth feeding on foliage of a wide range of herbaceous plants, both express a receptor ortholog, OR19, which shares 58% amino acid identity and 69% amino acid similarity. Following heterologous expression in the empty neuron system of *Drosophila melanogaster*, we show by single sensillum recordings that CpomOR19 and SlitOR19 show similar affinity to several substituted indanes. Tests with a series of compounds structurally related to 1-indanone show that 2-methyl-1-indanone, 2-ethyl-1-indanone, 3-methyl-1-indanone, and 1-indanone elicit a strong response from both ORs. A keto group in position 1 is essential for biological activity and so are both rings of the indane skeleton. However, there is an important difference in steric complementary of the indane rings and the receptor. Methyl substituents on the benzene ring largely suppressed the response. On the other hand, alkyl substituents at position 2 and 3 of the five-membered ring increased the response indicating a higher complementarity with the receptor cavity, in both CpomOR19 and SlitOR19. Our results demonstrate a conserved function of an odorant receptor in two moths that are phylogenetically and ecologically distant. It is conceivable that a conserved OR is tuned to signals that are relevant for both species, although their ecological roles are yet unknown. Our finding demonstrates that functional characterization of ORs leads to the discovery of novel semiochemicals that have not yet been found through chemical analysis of odorants from insects and their associated host plants.

Keywords: *Cydia pomonella*, *Spodoptera littoralis*, olfaction, olfactory receptor, 1-indanone, orthologous genes, structure activity relationships, functional characterization

INTRODUCTION

Perception of olfactory cues plays a fundamental role in insect life, and the olfactory system has evolved through adaptations to new environments, host, plant, and mate-finding signals (Bergstrom, 2008; Smadja and Butlin, 2009; Hansson and Stensmyr, 2011). Several studies have shown that the family of odorant receptor (OR) genes, which encode for proteins that detect and discriminate odorants, is highly divergent among insect taxa and even among closely related species (Jacquin-Joly and Merlin, 2004; Su et al., 2009; Engsontia et al., 2014; Depetris-Chauvin et al., 2015). This suggests that olfactory systems have evolved rapidly to enable perception of relevant odor signals. Selection drives the evolution of genes that facilitate host and mate finding, whereas behaviorally redundant OR genes are no longer expressed (Sánchez-Gracia et al., 2009; Hansson and Stensmyr, 2011; Suh et al., 2014; Andersson et al., 2015). Consequently, the insect OR repertoire is expected to be tuned to odor cues of ecological relevance, as indicated in the functional comparison between the OR repertoire of the vinegar fly, *Drosophila melanogaster,* and the malaria mosquito *Anopheles gambiae*, which shows little overlap (Hill et al., 2002; Carey et al., 2010; Suh et al., 2014; Karner et al., 2015). Orthologous ORs are of particular interest since may be tuned to odorants that are behaviorally and ecologically relevant across species (Bohbot et al., 2011).

Insect ORs identified so far generally show a low level of sequence conservation between species, ranging from 20 to 40% amino acid identity (Rützler and Zwiebel, 2005; Bohbot et al., 2007; Martin et al., 2011; Engsontia et al., 2014). A striking exception is the OR co-receptor, ORco, which shares 60–90% amino acid identity across different insect orders (Krieger et al., 2003; Larsson et al., 2004). A plausible reason for this conservation may lie in its function: ORco is an obligate co-receptor that forms a complex with ligand-selective ORs and is required for trafficking to olfactory neuron dendrites in all insects (Larsson et al., 2004; Jones et al., 2011). Apart from ORco, conserved ligand-selective ORs have been identified in closely related species. The OR2/OR10 clade of the mosquitoes *Aedes aegypti* and *An. gambiae* share 69% of amino acid identity and both respond strongly to indole, an important host signal for both species (Bohbot et al., 2011). Within Lepidoptera, several examples of conserved function for orthologous receptors have been reported, especially within the pheromone receptor family (de Fouchier et al., 2014; Jiang et al., 2014). There are clusters of ORs, however, that share high amino acid identity across species but whose function has not yet been elucidated; for example, OR18, a highly conserved receptor in six noctuid species, with an average of 88% amino acid identity (Brigaud et al., 2009).

A number of lepidopteran OR gene repertoires have been described, following genome and transcriptome sequencing (Jordan et al., 2009; Grosse-Wilde et al., 2011; Montagné et al., 2012, 2014; Cao et al., 2014; Gu et al., 2014; Liu et al., 2014; Corcoran et al., 2015; Yang et al., 2015; Zhang et al., 2015a,b). In our own transcriptome sequence analyses of the antennae of the codling moth (*Cydia pomonella*: Tortricidae; Bengtsson et al., 2012) and the cotton leafworm (*Spodoptera littoralis*: Noctuidae; Legeai et al., 2011; Jacquin-Joly et al., 2012; Poivet et al., 2013) we have identified one OR (OR19) with relatively high sequence similarity in both species. In *S. littoralis*, SlitOR19 was shown to be narrowly tuned to 1-indanone (de Fouchier et al, unpublished). We have compared the responses of SlitOR19 and its homolog CpomOR19 to 1-indanone, and its analogs, showing a similar response spectrum for these receptor orthologs in the codling moth and the African cotton leafworm. A qualitative structure-activity study of these receptors leads toward a better comprehension of the effect of amino acid sequence differences on OR tuning.

MATERIALS AND METHODS

Phylogenetic and Sequence Analysis

The previously described CpomOR19 amino acid sequence (Bengtsson et al., 2012) was used as a query in BLASTp search on the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). Among hits, putative ORs belonged to lepidopteran species only (*C. pomonella, S. littoralis, Bombyx mori, Heliothis virescens, Helicoverpa armigera, Helicoverpa assulta, Manduca sexta,* and *Danaus plexippus*). Sequences of the putative ORs retrieved were aligned with MAFFT, using the FFT-NS-2 algorithm with default parameters. A maximum likelihood tree was constructed with MEGA6 using the JTT+F algorithm with a bootstrap consensus inferred from 1000 replicates and Poisson correction of distances (Tamura et al., 2013).

The membrane topologies and transmembrane domains of CpomOR19 and SlitOR19 were predicted with five different prediction models-TMHMM (https://www.cbs.dtu.dk/services/TMHMM/), METSAM-SVM (http://bioinf.cs.ucl.ac.uk/psipred/), TOPCONS (http://topcons.cbr.su.se/), RHYTHM (http://proteinformatics.charite.de/rhythm/), and TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html).
From these, we selected the model that best fitted the OR characteristic structure (seven-transmembrane domains and extracellular C-terminus) and illustrated it with Protter (Omasits et al., 2014).

Heterologous Expression of Putative ORs in *Drosophila melanogaster*

The complete open reading frames (ORFs) encoding CpomOR19 and SlitOR19, from start codon to stop codon, were amplified by PCR, (CpomOR19: forward primer 5′-ATGTTTAGTTATGAAAATGAAGACAGC-3′, reverse primer 5′-TCAAGTCATTTCTTCAGTAGAGGT-3′; SlitOR19: forward primer 5′-ATGAAAAACCATTACATCTTGAA-3′, reverse primer 5′-TTACGAAGTTTGCGCATAAAAC-3′), using antennal cDNA synthetized with the RT-for-PCR kit (Invitrogen) as a template. For cloning of OR19 homologs, total RNA was extracted from 100 dissected antennae of mixed male and female 2–3 day old adult moths of each species. For extractions Trizol reagent (Invitrogen) was used according to manufacturer's standard protocol. After extraction, total RNA was purified via spin column purification with the RNeasy Mini Kit (Qiagen) according to manufacturer's standard protocol. Total RNA was used as template for first

strand cDNA synthesis with the RevertAid H minus Reverse Transcriptase kit, according to manufacturer's standard protocol. ORF sequence from start codon to stop codon of OR19 was PCR amplified from the cDNA. The purified PCR products were then cloned into the PCR8/GW/TOPO plasmid (Invitrogen), after which One Shot TOP10 cells were transformed (Invitrogen), and plated for overnight growth on Spectinomycin selective lysogeny broth (LB) growth plates. Colonies were assayed for the presence of the relevant insert in the correct orientation by PCR using either the forward gene specific primer (GSP) together with the M13 reverse primer, or the reverse GSP together with the M13 forward primer. Plasmids were purified by Miniprep (Qiagen), and then sequenced to confirm the presence and integrity of the OR inserts. The cassettes with the inserts were then transferred from the PCR8/GW/TOPO plasmid into the destination injection vector (pUASg-HA.attB) constructed by E. Furger and J. Bischof, kindly provided by the Basler group, Zürich (Bischof et al., 2007), using the Gateway LR Clonase II kit (Invitrogen). The destination vector with the correct insert (as confirmed by sequencing) was transformed into One Shot TOP10 cells (Invitrogen). Resultant colonies were cultured in 20 ml of LB media with Ampicillin and purified by Midiprep (Qiagen); the integrity and orientation of the inserts was confirmed by sequencing. Transformant UAS-CpomOR19 and UAS-SlitOR19 lines were generated by BestGene (Chino Hills, CA, USA) and Fly Facility (Clermont-Ferrand, France), respectively, using the PhiC31 integrase system. Briefly, recombinant pUASg-HA.attB-CpomOR19 and –SlitOR19 plasmids were injected into embryos of a *D. melanogaster* line containing an attP insertion site within the third chromosome (genotype y1 M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP}ZH-86Fb), leading to non-random integration; the transgenes were then crossed into the Δhalo mutant background. To drive expression of CpomOR19 and SlitOR19 in OSNs housed in the ab3 basiconic sensilla, the described transgenic lines were crossed with Δhalo; OR22a-Gal4 mutant *D. melanogaster* (Dobritsa et al., 2003; Hallem et al., 2004).

Single Sensillum Recordings

Flies expressing either CpomOR19 or SlitOR19 in the A neuron of ab3 basiconic sensilla were tested by single sensillum recordings (SSRs). Flies were restrained as described in Stensmyr et al. (2003). Briefly, flies were trapped inside 100 μl pipette tips with only the top half of the head protruding. A glass capillary was used to push the left antenna onto a piece of double-sided adhesive tape placed on a piece of glass. Both the pipette tip and the piece of glass with the antennae were mounted and fixed with dental wax on a microscope slide. Tungsten electrodes (diameter 0.12 mm, Harvard Apparatus Ltd., Edenbridge, UK), were electrolytically sharpened with a saturated KNO_3 solution, and used to penetrate the eye and the sensilla of the flies. The recording electrode (introduced at the base of the sensilla) was maneuvered with a DC-3K micromanipulator equipped with a PM-10 piezo translator (Märzhäuser Wetzler GmbH, Germany). The reference electrode was manually inserted through the eye. The signal from the olfactory sensory neurons (OSNs) was amplified 10 times with a probe (INR-02, Syntech, Hilversum, the Netherlands), digitally converted through an IDAC-4-USB (Syntech) interface, visualized and analyzed with the software Autospike v. 3.4 (Syntech).

During the recording sessions, a constant flow of 0.65 m/s of charcoal-filtered and humidified air was delivered through a glass tube with the outlet 15 mm apart from the antenna. The panel of odorant stimuli was presented to the insect by blowing air through pipettes inserted in a lateral hole of the glass tube delivering the constant charcoal-filtered humidified air. The air puff was controlled with a stimulus controller (Syntech SFC-1/b) and consisted of a flow of 2.5 ml of air during 0.5 s.

Synthetic Compounds and Odorant Stimuli

To determine ligands detected by CpomOR19, initially a panel with a wide range of synthetic compounds was tested (**Table 1**). The list of compounds included general plant odors previously tested for deorphanization of SlitOR19 (de Fouchier et al, unpublished), codling moth pheromone components (Arn et al., 1985), and microbial odorants (Witzgall et al., 2012). Compounds were diluted in redistilled hexane (LabScan), acetone (Sigma-Aldrich), or paraffin oil (Merck) to a concentration of 10 μg/μl. Stimuli were prepared by applying 10 μl (100 μg) of the diluted test compounds to 1.5 × 1 cm pieces of filter paper placed inside disposable glass Pasteur pipettes (VWR International, Stockholm, Sweden). Pipette tips were placed on the end of the Pasteur pipettes to decrease evaporation of compounds. Control pipettes with only solvent (hexane, acetone, and paraffin oil) were also prepared.

To investigate structural activity relationships between 1-indanone and selected analogs, a second odorant panel was tested for flies expressing either CpomOR19 or SlitOR19 (**Figure 1**). Compounds eliciting significant response in comparison to the solvent were used for dose response experiments, the concentration of the test compounds ranged from 1 ng to 100 μg in decadic steps applied to the filter paper in the stimulus pipette. Comparisons between receptor-activating compounds were made after correction for differences in vapor pressure (Bengtsson et al., 1990).

Fresh filter papers were prepared before each recording session, and kept at −18°C until the start of the recording session. Only complete recording sessions of the entire set of test stimuli were evaluated, and only one screening or dose response session was performed per individual fly and on a single sensillum.

SSR responses were quantified by counting the number of spikes for 500 ms starting from the onset of the response (as determined by the earliest response for the recording session), subtracting the number of spikes during 500 ms before response. Five whole-panel screenings for ligands of CpomOR19 were performed, screenings of the panel of structurally related compounds were done five times for CpomOR19 and SlitOR19. For dose response experiments, eight replicates were carried out at each dose for each receptor.

Responses of CpomOR19 and SlitOR19 to the panel of structurally related compounds and dose response experiments

TABLE 1 | Responses of *D. melanogaster* flies expressing CpomOR19 to synthetic compounds tested at 100 μg on filter paper.

Compound class	Compound	Chemical purity (%)	Source	Spike frequency[a]
HYDROCARBONS				
Monoterpenes	α-Pinene	98	Aldrich	+
	β-Pinene	99	Fluka	+
	β-Myrcene	95	Fluka	
	β-Ocimene	90	Safc	
	3-Carene	95	Aldrich	
Sesquiterpenes	α-farnesene	99	Bedoukian	
	α-Copaene	98	Bedoukian	
	α-Humulene	98	Aldrich	+
	β-Caryophyllene	98.5	Aldrich	
Homoterpenes	TMTT[b]	98	Aldrich	+
	DMNT[c,d]	95		
ALCOHOLS				
Aliphatics	1-Hexanol	98	Aldrich	++
	1-Heptanol	99	Aldrich	
	1-Octanol	99.5	Aldrich	
	1-Nonanol	99.5	Aldrich	
	1-Tetradecanol	99	Fluka	+
	(Z)-3-Hexenol	98	Aldrich	+
	(E)-2-Hexenol	96	Aldrich	+
	Butyl alcohol	99.5	Sigma	+
	(E)-3-Hexen-1-ol	97	Aldrich	+
	Codlemone[e]	98.6		
	1-Dodecanol	98	Fluka	+
	(E)-9-Dodecenol	99	Farchan Labs Inc	+
Aromatics	Thymol	99.5	Aldrich	
	Carvacrol	98	Aldrich	+
	Eugenol	98	Aldrich	
	Estragol	96	Sigma	
Monoterpenes	Geraniol	98	Aldrich	
	Citronellol	95	Aldrich	
	± Linalool	97	Aldrich	+
Sesquiterpenes	*(E,E)*-Farnesol	95	Aldrich	+
	± Nerolidol	98	Aldrich	+
Diterpenes	Phytol	99	Aldrich	
ALDEHYDES				
Aliphatics	*(E)*-2-Hexenal	98	Aldrich	+
	Nonanal	95	Aldrich	
	Decanal	99	Aldrich	
Aromatics	Phenyl acetaldehyde	98	Aldrich	
	Benzaldehyde	99.5	Aldrich	
ETHERS				
Aromatics	Benzyl methyl ether	98	Aldrich	
ESTERS				
Aliphatics	*(Z)*-3-Hexenyl acetate	98	Aldrich	
	Butyl butyrate	99	Aldrich	
	Methyl hexanoate	99	Aldrich	+
	Hexyl butyrate	98	Aldrich	
	Methyl jasmonate	98	Aldrich	
	Propyl hexanoate	99	Aldrich	+
	Pear ester	98	Aldrich	+
	Isoamyl acetate	95	Aldrich	+
	Isobutyl acetate	99	Aldrich	+
	Codlemone acetate	97	Bedoukian	+
	Hexyl propionate	97	Aldrich	+
	Butyl acetate	99	Aldrich	+
Aromatics	Methyl salicylate	99	Sigma	+
	Methyl benzoate	99	Aldrich	+
	2-Phenylethyl acetate	99	Aldrich	
KETONES				
Aliphatics	Geranyl acetone	96	Aldrich	+
	(Z)-Jasmone[d]	98		
	2-Heptanone	98	Aldrich	+
	Sulcatone	98	Aldrich	+
Aromatics	Acetophenone	99	Acros	+
	1-indanone	99	Aldrich	+++
ACIDS				
Aliphatics	Acetic acid	99	Aldrich	
OTHERS				
	Indole	99	Aldrich	

[a] Spike frequency (Hz) is used as measure of response strength: 1–10 Hz (+), 11–49 Hz (++), >50 Hz (+++).
[b] (E,E) 4,8,12-trimethyltrideca-1,3,7,11-tetraene.
[c] (E)-4,8-dimethyl-1,3,7-nonatriene.
[d] Gift from Prof. Wittko Francke.
[e] Gift from Prof. Heinrich Arn.

were compared with Two-way ANOVA with repeated measures, followed by LSD *post-hoc* test. All statistical analyses were performed using SPSS Version 19.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Phylogeny and Sequence Analysis

Comparison of protein sequences of putative orthologs from different lepidopteran species showed that the receptors OR21 and OR22 of *B. mori*, along with OR19 of *S. littoralis*, *H. virescens,* and *C. pomonella* cluster within one group (**Figure 2**). Among these sequences, SlitOR19 shared the highest amino acid identity (58%) with CpomOR19, while the others share 42–55% (**Figure 3A**). According to receptor topology prediction (OCTOPUS algorithm, TOPCONS), the main differences between the two sequences were observed in the putative extracellular C-terminus which SlitOR19 has a four residues shorter sequence, along with the addition of residues in two regions, one located in the fourth transmembrane domain (M) and the other in the third intracellular loop (RPKSAP). However, most of the non-conservative point mutations correlated to substitutions in

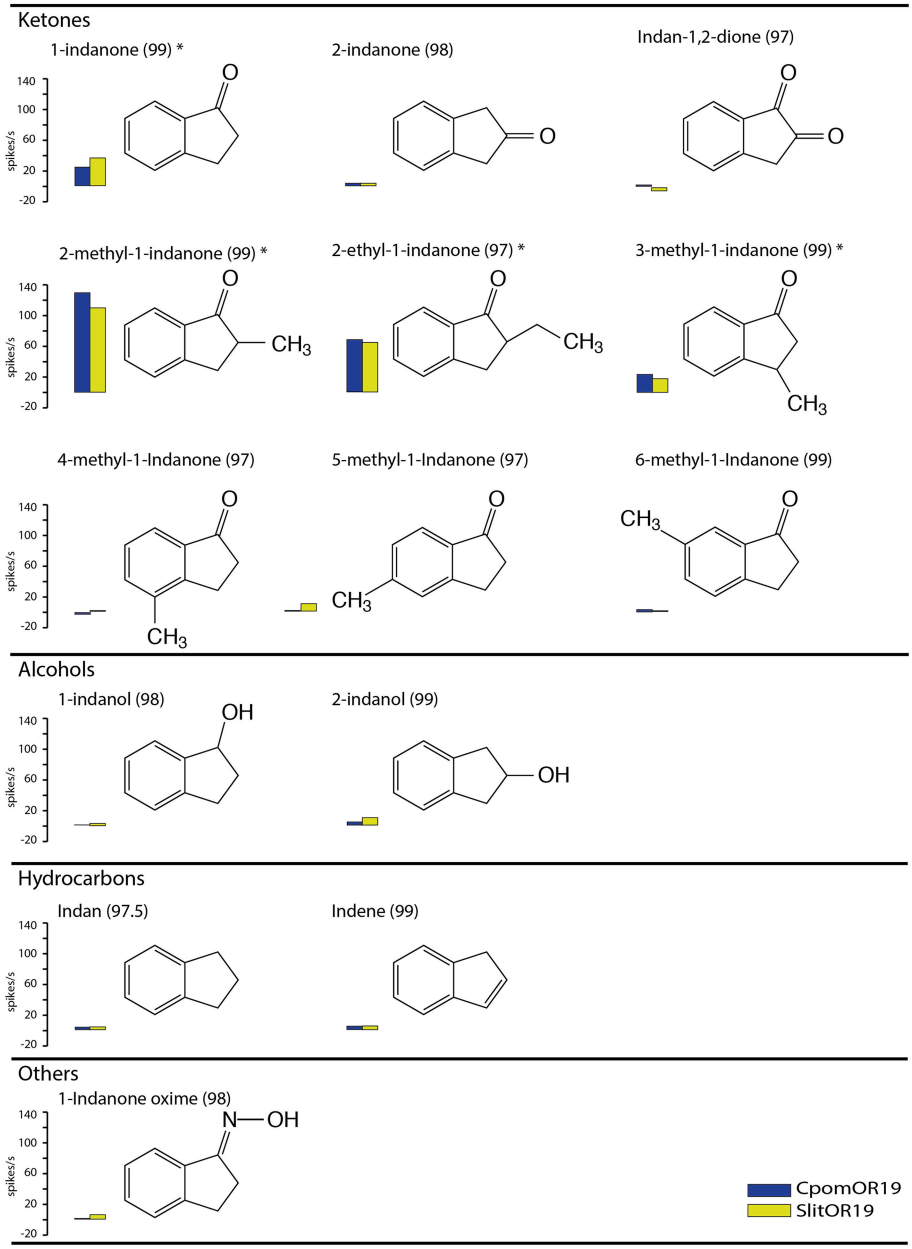

FIGURE 1 | Response profiles of CpomOR19 and SlitOR19 to 1-indanone and structurally related compounds at 100 μg on filter paper. Asterisks denote significant differences between the response elicited by the indicated compound and the solvent at $P < 0.05$ (Two-way ANOVA with repeated measures, LSD *post-hoc* test, $n = 5$). Chemical purity is shown in brackets, compounds were purchased from Aldrich.

the first transmembrane region and in the cytoplasmic side (loop 2), while only a few mutations are predicted to be located on the extracellular side (**Figure 3B**).

Selectivity of CpomOR19 toward Putative Ligands

SSR recordings from ab3A OSN of *D. melanogaster* that expressed CpomOR19 showed that of 64 stimuli tested at the maximum dose of 100 μg loaded on filter paper, only 1-indanone elicited a strong electrophysiological response (>50 Hz; **Table 1**, **Supplementary Figure 1**).

Effect of Chemical Structure on Specificity and Sensitivity of CpomOR19 and SlitOR19

When tested at the maximum dose of 100 μg, the responses of CpomOR19 and SlitOR19 did not differ significantly between them for any of the indanone analogs tested. Besides 1-indanone, both ORs responded to three of the other 13 compounds tested.

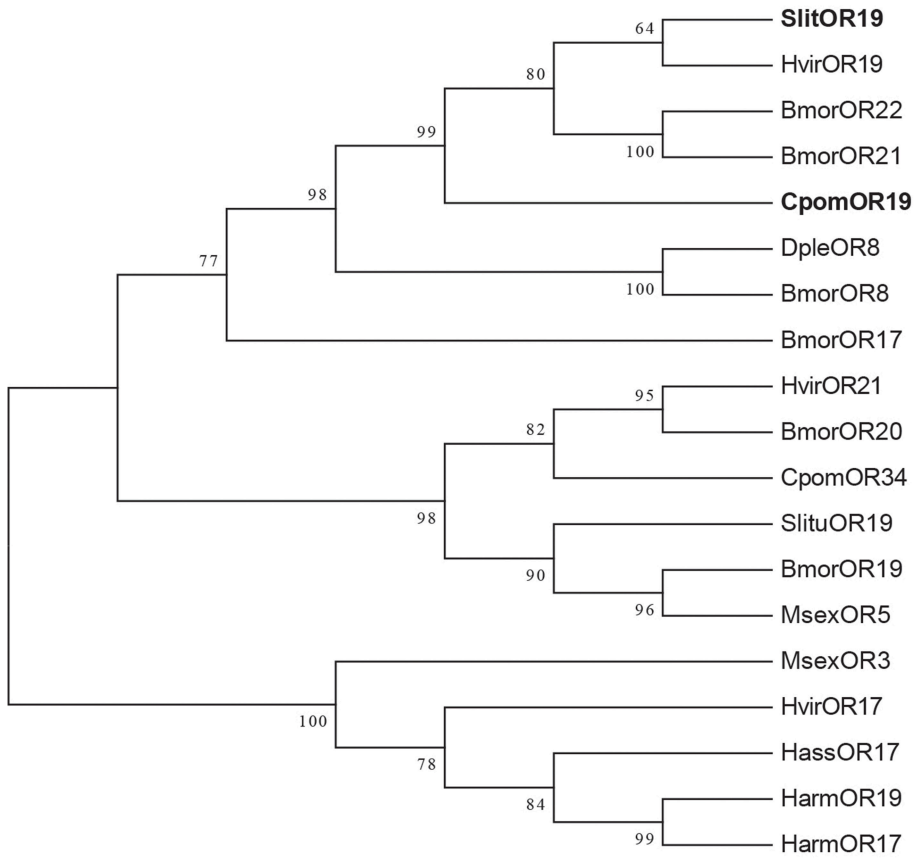

FIGURE 2 | Maximum-likelihood tree of the sequences of *Cydia pomonella* CpomOR19 and homologs from other lepidopteran species. The distance tree is calculated by MEGA6 based on sequence alignment using MAFFT. Branch support is shown for values above 60%. *C. pomonella* (Cpom), *B. mori* (Bmor), *S. littoralis* (Slit), *S. litura* (Slitu), *H. virescens* (Hvir), *D. plexippus* (Dple), *M. sexta* (Msex), *H. assulta* (Hass), *H. armigera* (Harm).

The strongest responses were elicited by 2-methyl-1-indanone and 2-ethyl-1-indanone, followed by 1-indanone and 3-methyl-1-indanone (**Figure 1**).

Dose-response experiments also revealed that both CpomOR19 and SlitOR19 had a lower threshold for 2-methyl-1-indanone and 2-ethyl-1-indanone, reacting to lower amounts of these than to 1-indanone and 3-methyl-1-indanone (**Figure 4**). For 2-methyl-1-indanone, 1 μg on the filter paper was sufficient to elicit a significant response in comparison to the solvent and with correction for differences in vapor pressure taken into account, 2-ethyl-indanone elicited above-threshold responses at quantities below 1 μg. The only significant discrepancy between the two receptors was observed in CpomOR19 that responded more strongly to 3-methyl-indanone than SlitOR19 at a dose of 10 μg.

DISCUSSION

Codling moth *C. pomonella* (Lepidoptera, Tortricidae) and African cotton leafworm *S. littoralis* (Lepidoptera, Noctuidae) share two orthologous ORs with conserved function, CpomOR19 and SlitOR19 (**Figures 1**, **2**). Furthermore, SlitOR19 and CpomOR19 are expressed in adults of both sexes of *S. littoralis* and *C. pomonella* (Bengtsson et al., 2012; Poivet et al., 2013). This is an intriguing finding: in addition to taxonomic position (Kristensen et al., 2007), the two species differ with respect to host plant and feeding habit. *C. pomonella* larvae mine in apple and pear fruit, or in walnuts, whereas *S. littoralis* feeds on the leaves of a very wide range of herbaceous plants (Salama et al., 1971; Bradley et al., 1979). The occurrence of receptors with conserved function and their similar expression patterns likely reflect a role of one or more substituted indanone compounds in the behavioral ecology of these two species.

Structurally and Functionally Conserved ORs

Sequence similarity is not a reliable indicator of OR function. However, our results show that the response profiles of CpomOR19 and SlitOR19, with 58% amino acid identity, are virtually the same: both respond to 1-indanone and structurally related compounds (**Figures 1**, **3A**). Similarly, pheromone receptors from heliothinae moths, HarmOR14b, HassOR16 and HvirOR6, with amino acid identities between 53 and 65%, all responded to (*Z*)-9-tetradecenal (Jiang et al., 2014). In contrast,

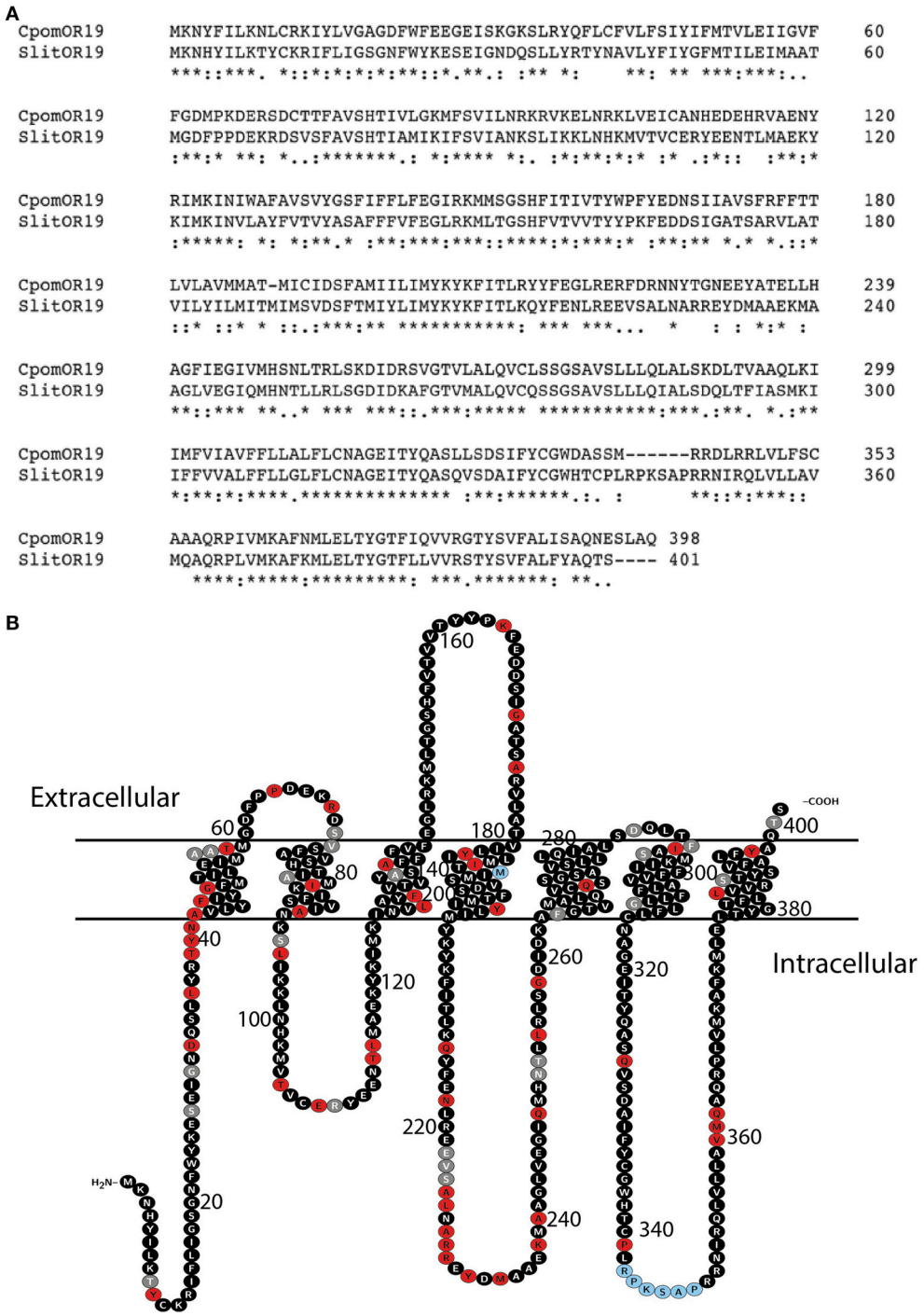

FIGURE 3 | (A) Amino acid alignment of CpomOR19 and SlitOR19. Amino acid sequence differences are indicated as highly (:) and moderately (.) conservative substitutions and non-conservative substitutions (blanks), while asterisks indicate identity across both sequences. **(B)** Putative protein topology of SlitOR19 and its differences with CpomOR19. Gray dots indicate moderately conservative substitutions, red dots indicate non-conservative substitution of residues and light blue dots indicate addition of residues in SlitOR19 as compared to CpomOR19.

a single mutation is enough to change the specificity of a sex pheromone receptor between two species of *Ostrinia* (Leary et al., 2012), demonstrating that minor changes in amino acid sequences can lead to conformational changes in membrane proteins that have profound effects on OR function, specificity and sensitivity (Curran and Engelman, 2003; Hopf et al., 2015).

For CpomOR19 and SlitOR19, most of the non-conserved mutations were found on the first transmembrane region

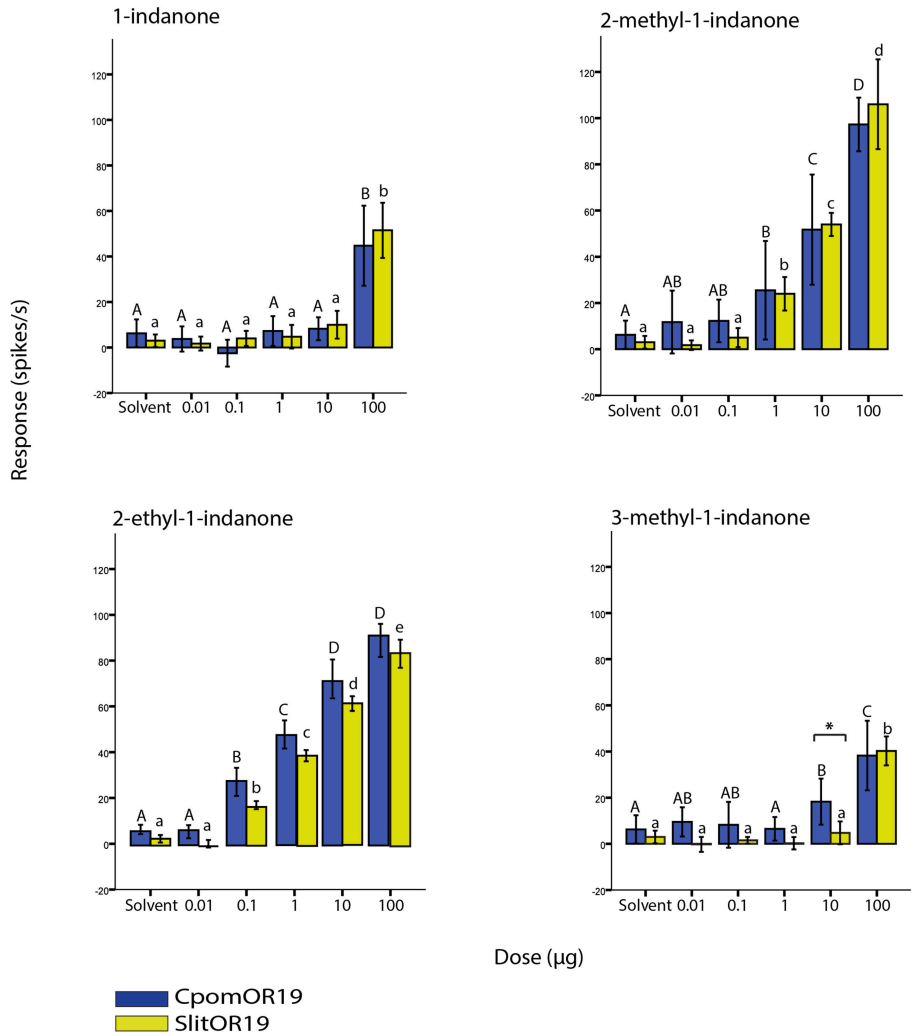

FIGURE 4 | Dose-dependent responses of CpomOR19 and SlitOR19 to 1-indanone and structurally related compounds. Responses to 2-ethyl-1-indanone are adjusted to account for differences in vapor pressure. Bars of the same color followed by different letters indicate subgroups with statistically significant differences. Asterisk denotes significant differences between species for the dose indicated at $P < 0.01$ (Two-way ANOVA with repeated measures, LSD *post-hoc* test, $n = 8$).

and on the intracellular loop 2 of the predicted proteins (**Figure 3B**). Hopf et al. (2015) showed that the N-terminus tail, the extracellular loop 2 and the intracellular loop 3, are kept under strong evolutionary constraint, indicating their functional importance in receptors of *D. melanogaster*. Point mutations within the third and sixth transmembrane regions can affect the sensitivity and selectivity of ORs, as demonstrated by Steinwender et al. (2015) for the pheromone receptor OR7 of *Ctenopseustis oblicuana* and *Ctenopseustis herana,* and may drive speciation events. In CpomOR19 and SlitOR19, these regions show only minor changes, except a deletion of the final four residues of the C-terminus sequence of SlitOR19. However, this deletion did not affect OR tuning, compared with CpomOR19. In contrast, Hill et al. (2015) recently demonstrated that a deletion of the C-terminus in one of the two paralogous ORs in the mosquito *Culex quinquefasciatus* has a profound effect on enantiomeric selectivity. The specific mechanisms governing OR functions remain, however, unknown. It therefore cannot not be excluded that non-conservative mutations concern even functional sites: amino acid interactions, which appear to strongly affect functional properties, may restore receptor tuning.

CpomOR19 and SlitOR19 are Tuned to 1-Indanones

Among the first panel of odorants 1-indanone elicited the strongest response (**Table 1**). Ensueing tests with a number of structurally related 1-indanone analogs showed that the affinity of both ORs to 2-methyl-1-indanone and 2-ethyl-1-indanone was even higher (**Figures 1**, **4**).

Analysis of the molecular receptive range of CpomOR19 and SlitOR19 provides insight into their interaction with odorant ligands. For both ORs, the nature and position of the functional group and the presence and position of methyl and ethyl substituents all affected receptor-ligand interactions.

A carbonyl group in position 1 is required for biological activity, as demonstrated by the lack of response toward alcohols, hydrocarbons and an imine. This is in agreement with Liljefors et al. (1984), showing that the functional group plays an essential role in successful ligand-OR interactions. Acetophenone, a substance which interacts with the receptor through both the carbonyl group and the benzene ring at the same position in space as 1-indanone, did not elicit an OR response. We therefore deduce that the five-membered ring of the indane skeleton is required for biological activity. Finally, a complete lack of response to indan-1,2-dione indicates that the polarity and electron distribution of the additional keto-group intervene and prevent the molecule from binding to the OR. By introducing alkyl substituents as space-probes at different positions of the indane structure, we were able to characterize the degree of complementarity between this part of the substrate and the receptor. A similar approach was taken by Jönsson et al. (1992) to study the interaction of a moth sex pheromone with its receptor cell. Addition of a methyl and ethyl group to the second carbon of the five-membered ring increases the response. This suggests the alkyl group interacts with a complementary receptor site within the OR, that could consist of a hydrophobic "pocket." Our results also indicate that the addition of methyl space-probe groups to the benzene ring (4-, 5- and 6-methyl-1-indanone) decreased biological activity. We hypothesize that these additions caused repulsive, steric interference between the analog and a complementary receptor site of the OR.

Earlier analyses of the molecular receptive range of ORs by electrophysiological recordings from native olfactory sensory neurons (OSNs) support our findings. For example, Stranden et al. (2003) demonstrated structural-activity relationships in the electrophysiological responses of three heliothine moths to the sesquiterpene germacrene D. The selective response of these OSNs to germacrene D was defined by the ten-membered ring system, the position of three double bonds and the position of the isopropyl group. Research on pheromone receptors of the moth *Agrotis segetum* has also shown that changes in shape and bulkiness, length, position of the double bond or nature of the functional group of the (*Z*)-5-decenyl acetate molecule (one of the three pheromone components of this species), have an effect, direct or indirect, on the interaction of the molecules with the receptor binding sites. Here, the acetate group, the double bond and the terminal alkyl chain are the three molecular parts which are most likely responsible for the selectivity of the receptor (Bengtsson et al., 1987, 1990; Jönsson et al., 1991).

The response to the indanone analogs was overall similar for CpomOR19 and SlitOR19, although significant differences were observed in dose-response relationships to 3-methyl-indanone (**Figure 4**). This response shift may be due to residue substitutions. Further experiments, for example including ORs with induced point mutations, are required to reveal the basis of these differences.

The Ecological Role of Indanes is Yet Unknown

Semiochemicals are natural compounds which elicit a behavioral response, and which activate dedicated ORs at low concentrations (Bohbot and Dickens, 2012). *Spodoptera* larval frass, which deters oviposition in conspecific females, contains 1-indanone (Klein et al., 1990; Anderson et al., 1993), but we were unable to corroborate presence of 1-indanone or any other indane in frass collections of *S. littoralis* reared on several diets (data not shown). Indanone is found in roots of tropical plants (Okpekon et al., 2009), decaying wood fungi (Rukachaisirikul et al., 2013), and filamentous marine cyanobacteria (Nagle et al., 2000), which are probably not relevant for *S. littoralis* or *C. pomonella*. However, our results indicate that one or several indanone analogs are ligands for CpomOR19 and SlitOR19, but the source of these compounds and their behavioral and ecological roles are yet to be elucidated.

Pterosins are a group of natural compounds, composed of modified 2-methyl-1-indanones (Syrchina and Semenov, 1982). Pterosins are produced by the fern *Pteridium aquilinum* and are known to be toxic and show anti-feeding effects in various insects (Jones and Firn, 1979). These compounds make good candidates for ligands of CpomOR19 and SlitOR19 since they are similar in structure to 2-methyl-1-indanone, which elicited one of the strongest responses in our screening. Unfortunately we were unable to test pterosins, because they are not commercially available and we did not screen plants producing them. To our knowledge, pterosins are not produced by other plants and ferns are not commonly found in *C. pomonella* and *S. littoralis* habitats, but structurally similar compounds may occur in their host or non-host plants. Further research on plant or insect chemical profiles, together with behavioral studies of substituted indanes, is needed to identify the natural, key ligands for OR19 and to decipher their ecological relevance.

The olfactory and behavioral responses of codling moth and cotton leafworm to host and non-host plants have been studied thoroughly (Bäckman et al., 2001; Bengtsson et al., 2001, 2014; Witzgall et al., 2005; Trona et al., 2010, 2013; Saveer et al., 2012; Binyameen et al., 2013, 2014; Borrero-Echeverry et al., 2015). Our study accentuates that analytical chemistry of current, known host plant associations provides an incomplete pool of compounds for the identification of the ligands mediating insect olfactory behavior. Our comparison of an ortholog OR in *C. pomonella* and *S. littoralis* validates functional characterization of OR repertoires as an alternative approach, leading to a more complete description of the olfactory system.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2015.00131

Supplementary Figure 1 | Response profile of CpomOR19 to synthetic compounds tested at 100 μg on filter paper (mean ± SE, $n = 5$).

REFERENCES

Anderson, P., Hilker, M., Hansson, B., Bombosch, S., Klein, B., and Schildknecht, H. (1993). Oviposition deterring components in larval frass of *Spodoptera littoralis* (Boisd.) (*Lepidoptera: Noctuidae*): a behavioral and electrophysiological evaluation. *J. Insect Physiol.* 39, 129–137. doi: 10.1016/0022-1910(93)90104-Y

Andersson, M. N., Löfstedt, C., and Newcomb, R. D. (2015). Insect olfaction and the evolution of receptor tuning. *Front. Ecol. Evol.* 3:53. doi: 10.3389/fevo.2015.00053

Arn, H., Guerin, P., Buser, H., Rauscher, S., and Mani, E. (1985). Sex pheromone blend of the codling moth, *Cydia pomonella*: evidence for a behavioral role of dodecan-1-ol. *Experientia* 41, 1482–1484. doi: 10.1007/BF01950048

Bäckman, A. C., Bengtsson, M., Borg-Karlsson, A. K., Liblikas, I., and Witzgall, P. (2001). Volatiles from apple eliciting antennal responses in female codling moth *Cydia pomonella* (L.) (*Lepidoptera: Tortricidae*): effect of plant injury and sampling technique. *Z. Naturforsch.* C 56, 262–268. doi: 10.1515/znc-2001-3-415

Bengtsson, J. M., Gonzalez, F., Cattaneo, A. M., Montagné, N., Walker, W. B., Bengtsson, M., et al. (2014). A predicted sex pheromone receptor of codling moth *Cydia pomonella* detects the plant volatile pear ester. *Front. Ecol. Evol.* 2:33. doi: 10.3389/fevo.2014.00033

Bengtsson, J. M., Trona, F., Montagné, N., Anfora, G., Ignell, R., Witzgall, P., et al. (2012). Putative chemosensory receptors of the codling moth, *Cydia pomonella,* identified by antennal transcriptome analysis. *PLoS ONE* 7:e31620. doi: 10.1371/journal.pone.0031620

Bengtsson, M., Bäckman, A.-C., Liblikas, I., Ramirez, M. I., Borg-Karlson, A.-K., Ansebo, L., et al. (2001). Plant odor analysis of apple: antennal response of codling moth females to apple volatiles during phenological development. *J. Agric. Food Chem.* 49, 3736–3741. doi: 10.1021/jf0100548

Bengtsson, M., Liljefors, T., and Hansson, B. S. (1987). Dienic analogs of (Z)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*: synthesis, conformational analysis and structure-activity relationships. *Bioorg. Chem.* 15, 409–422. doi: 10.1016/0045-2068(87)90036-8

Bengtsson, M., Liljefors, T., Hansson, B. S., Löfstedt, C., and Copaja, S. V. (1990). Structure-activity relationships for chain-shortened analogs of (Z)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*. *J. Chem. Ecol.* 16, 667–684. doi: 10.1007/BF01016478

Bergstrom, L. G. W. (2008). Chemical communication by behavior-guiding olfactory signals. *Chem. Commun.* 34, 3959–3979. doi: 10.1039/b712681f

Binyameen, M., Hussain, A., Yousefi, F., Birgersson, G., and Schlyter, F. (2013). Modulation of reproductive behaviors by non-host volatiles in the polyphagous Egyptian cotton leafworm, *Spodoptera littoralis. J. Chem. Ecol.* 39, 1273–1283. doi: 10.1007/s10886-013-0354-4

Binyameen, M., Anderson, P., Ignell, R., Birgersson, G., Razaq, M., Shad, S. A., et al. (2014). Identification of plant semiochemicals and characterization of new olfactory sensory neuron types in a polyphagous pest moth, *Spodoptera littoralis*. *Chem. Senses* 39, 719–733. doi: 10.1093/chemse/bju046

Bischof, J., Maeda, R. K., Hediger, M., Karch, F., and Basler, K. (2007). An optimized transgenesis system for *Drosophila* using germ-line-specific φC31 integrases. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3312–3317. doi: 10.1073/pnas.0611511104

Bohbot, J. D., and Dickens, J. C. (2012). Selectivity of odorant receptors in insects. *Front. Cell. Neurosci.* 6:29. doi: 10.3389/fncel.2012.00029

Bohbot, J. D., Jones, P. L., Wang, G., Pitts, R. J., Pask, G. M., and Zwiebel, L. J. (2011). Conservation of indole responsive odorant receptors in mosquitoes reveals an ancient olfactory trait. *Chem. Senses* 36, 149–160. doi: 10.1093/chemse/bjq105

Bohbot, J., 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. doi: 10.1111/j.1365-2583.2007.00748.x

Borrero-Echeverry, F., Becher, P. G., Birgersson, G. Å. O., Bengtsson, M., Witzgall, P., and Saveer, A. M. (2015). Flight attraction of *Spodoptera littoralis* (*Lepidoptera, Noctuidae*) to cotton headspace and synthetic volatile blends. *Front. Ecol. Evol.* 3:56. doi: 10.3389/fevo.2015.00056

Bradley, J. D., Tremewan, W. G., and Smith, A. (1979). *British Tortricoid Moths. Tortricidae: Olethreutinae*. London: The Ray Society.

Brigaud, I., Montagné, N., Monsempes, C., François, M. C., and Jacquin-Joly, E. (2009). Identification of an atypical insect olfactory receptor subtype highly conserved within noctuids. *FEBS J.* 276, 6537–6547. doi: 10.1111/j.1742-4658.2009.07351.x

Cao, D., Liu, Y., Wei, J., Liao, X., Walker, W. B., Li, J., et al. (2014). Identification of candidate olfactory genes in *Chilo suppressalis* by antennal transcriptome analysis. *Int. J. Biol. Sci.* 10, 846–860. doi: 10.7150/ijbs.9297

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. doi: 10.1038/nature08834

Corcoran, J. A., Jordan, M. D., Thrimawithana, A. H., Crowhurst, R. N., and Newcomb, R. D. (2015). The peripheral olfactory repertoire of the lightbrown apple moth, *Epiphyas postvittana*. *PLoS ONE* 10:e0128596. doi: 10.1371/journal.pone.0128596

Curran, A. R., and Engelman, D. M. (2003). Sequence motifs, polar interactions and conformational changes in helical membrane proteins. *Curr. Opin. Struct. Biol.* 13, 412–417. doi: 10.1016/S0959-440X(03)00102-7

de Fouchier, A., Mirabeau, O., Montagné, N., and Jacquin-Joly, E. (2014). "Current views on the function and evolution of lepidopteran olfactory receptors," in *Short Views on Insect Biochemistry and Molecular Biology*, Chap. 17, Vols. 1, 2, eds R. Chandrasekar, B. K. Tyagi, Z. Z. Gui, and G. R. Reeck (Manhattan, NY: International Book Mission; Academic Publisher), 385–408.

Depetris-Chauvin, A., Galagovsky, D., and Grosjean, Y. (2015). Chemicals and chemoreceptors: ecologically relevant signals driving behavior in *Drosophila*. *Front. Ecol. Evol.* 3:41. doi: 10.3389/fevo.2015.00041

Dobritsa, A. A., van der Goes van Naters, W., Warr, C. G., Steinbrecht, R. A., and Carlson, J. R. (2003). Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron* 37, 827–841. doi: 10.1016/S0896-6273(03)00094-1

Engsontia, P., Sangket, U., Chotigeat, W., and Satasook, C. (2014). Molecular evolution of the odorant and gustatory receptor genes in lepidopteran insects: implications for their adaptation and speciation. *J. Mol. Evol.* 79, 21–39. doi: 10.1007/s00239-014-9633-0

Grosse-Wilde, E., Kuebler, L. S., Bucks, S., Vogel, H., Wicher, D., and Hansson, B. S. (2011). Antennal transcriptome of *Manduca sexta*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 7449–7454. doi: 10.1073/pnas.1017963108

Gu, S. H., Sun, L., Yang, R. N., Wu, K. M., Guo, Y. Y., Li, X. C., et al. (2014). Molecular characterization and differential expression of olfactory genes in the antennae of the black cutworm moth *Agrotis ipsilon*. *PLoS ONE* 9:e103420. doi: 10.1371/journal.pone.0103420

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. doi: 10.1016/j.cell.2004.05.012

Hansson, B. S., and Stensmyr, M. C. (2011). Evolution of insect olfaction. *Neuron* 72, 698–711. doi: 10.1016/j.neuron.2011.11.003

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. doi: 10.1126/science.1076196

Hill, S. R., Majeed, S., and Ignell, R. (2015). Molecular basis for odorant receptor tuning: a short C-terminal sequence is necessary and sufficient for selectivity of mosquito Or8. *Insect Mol. Biol.* 24, 491–501. doi: 10.1111/imb.12176

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. doi: 10.1038/ncomms7077

Jacquin-Joly, E., Legeai, F., Montagné, N., Monsempes, C., François, M.-C., Poulain, J., et al. (2012). Candidate chemosensory genes in female antennae of the noctuid moth *Spodoptera littoralis*. *Int. J. Biol. Sci.* 8, 1036. doi: 10.7150/ijbs.4469

Jacquin-Joly, E., and Merlin, C. (2004). Insect olfactory receptors: contributions of molecular biology to chemical ecology. *J. Chem. Ecol.* 30, 2359–2397. doi: 10.1007/s10886-004-7941-3

Jiang, X.-J., Guo, H., Di, C., Yu, S., Zhu, L., Huang, L.-Q., et al. (2014). Sequence similarity and functional comparisons of pheromone receptor orthologs in two closely related *Helicoverpa* species. *Insect Biochem. Mol. Biol.* 48, 63–74. doi: 10.1016/j.ibmb.2014.02.010

Jones, C. G., and Firn, R. D. (1979). Resistance of *Pteridium aquilinum* to attack by non-adapted phytophagous insects. *Biochem. Syst. Ecol.* 7, 95–101. doi: 10.1016/0305-1978(79)90015-2

Jones, P. L., Pask, G. M., Rinker, D. C., and Zwiebel, L. J. (2011). Functional agonism of insect odorant receptor ion channels. *Proc. Natl. Acad. Sci. U.S.A.* 108, 8821–8825. doi: 10.1073/pnas.1102425108

Jönsson, S., Liljefors, T., and Hansson, B. S. (1991). Alkyl substitution in terminal chain of (Z)-5-decenyl acetate, a pheromone component of turnip moth, *Agrotis segetum*. Synthesis, single-sensillum recordings, and structure-activity relationships. *J. Chem. Ecol.* 17, 103–122. doi: 10.1007/BF00994425

Jönsson, S., Liljefors, T., and Hansson, B. S. (1992). Introduction of methyl groups to acetate substituted chain of (Z)-5-decenyl acetate, a pheromone component of turnip moth, *Agrotis segetum*. Synthesis, single-sensillum recordings, and structure-activity relationships. *J. Chem. Ecol.* 18, 637–657. doi: 10.1007/BF00987825

Jordan, M. D., Anderson, A., Begum, D., Carraher, C., Authier, A., Marshall, S. D., et al. (2009). Odorant receptors from the light brown apple moth (*Epiphyas postvittana*) recognize important volatile compounds produced by plants. *Chem. Senses* 34, 383–394. doi: 10.1093/chemse/bjp010

Karner, T., Schneider, I., Schultze, A., Breer, H., and Krieger, J. (2015). Co-expression of six tightly clustered odorant receptor genes in the antenna of the malaria mosquito *Anopheles gambiae*. *Front. Ecol. Evol.* 3:26. doi: 10.3389/fevo.2015.00026

Klein, B., Schildknecht, H., Hilker, M., and Bombosch, S. (1990). Eiablagehemmende wirkstoffe aus dem Larvenkot von *Spodoptera littoralis* (Boisd.). *Z. Naturforsch.* 45, 895–901.

Krieger, J., Klink, O., Mohl, C., Raming, K., and Breer, H. (2003). A candidate olfactory receptor subtype highly conserved across different insect orders. *J. Comp. Physiol. A* 189, 519–526. doi: 10.1007/s00359-003-0427-x

Kristensen, N. P., Scoble, M. J., and Karsholt, O. (2007). Lepidoptera phylogeny and systematics: the state of inventorying moth and butterfly diversity. *Zootaxa* 1668, 699–747.

Larsson, M. C., Domingos, A. I., Jones, W. D., Chiappe, M. E., Amrein, H., and Vosshall, L. B. (2004). Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* 43, 703–714. doi: 10.1016/j.neuron.2004.08.019

Leary, G. P., Allen, J. E., Bunger, P. L., Luginbill, J. B., Linn, C. E. Jr, Macallister, I. E., et al. (2012). Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14081–14086. doi: 10.1073/pnas.1204661109

Legeai, F., Malpel, S., Montagné, N., Monsempes, C., Cousserans, F., Merlin, C., et al. (2011). An expressed sequence tag collection from the male antennae of the noctuid moth *Spodoptera littoralis*: a resource for olfactory and pheromone detection research. *BMC Genomics* 12:86. doi: 10.1186/1471-2164-12-86

Liljefors, T., Thelin, B., and Van Der Pers, J. N. C. (1984). Structure activity relationships between stimulus molecule and response of a pheromone receptor cell in turnip moth, *Agrotis segetum* – modification of the acetate group. *J. Chem. Ecol.* 10, 1661–1675. doi: 10.1007/BF00987353

Liu, N. Y., Xu, W., Papanicolaou, A., Dong, S. L., and Anderson, A. (2014). Identification and characterization of three chemosensory receptor families in the cotton bollworm Helicoverpa armigera. *BMC Genomics* 15:597. doi: 10.1186/1471-2164-15-597

Martin, J. P., Beyerlein, A., Dacks, A. M., Reisenman, C. E., Riffell, J. A., Lei, H., et al. (2011). The neurobiology of insect olfaction: sensory processing in a comparative context. *Progr. Neurobiol.* 95, 427–447. doi: 10.1016/j.pneurobio.2011.09.007

Montagné, N., Chertemps, T., Brigaud, I., François, A., François, M. C., de Fouchier, A., et al. (2012). Functional characterization of a sex pheromone receptor in the pest moth *Spodoptera littoralis* by heterologous expression in *Drosophila*. *Eur. J. Neurosci.* 36, 2588–2596. doi: 10.1111/j.1460-9568.2012.08183.x

Montagné, N., de Fouchier, A., Newcomb, R. D., and Jacquin-Joly, E. (2014). Advances in the identification and characterization of olfactory receptors in insects. *Prog. Mol. Biol. Transl. Sci.* 130, 55–80. doi: 10.1016/bs.pmbts.2014.11.003

Nagle, D. G., Zhou, Y.-D., Park, P. U., Paul, V. J., Rajbhandari, I., Duncan, C. J., et al. (2000). A new indanone from the marine cyanobacterium *Lyngbya majuscula* that inhibits hypoxia-induced activation of the VEGF promoter in Hep3B cells. *J. Nat. Prod.* 63, 1431–1433. doi: 10.1021/np000216e

Okpekon, T., Millot, M., Champy, P., Gleye, C., Yolou, S., Bories, C., et al. (2009). A novel 1-indanone isolated from *Uvaria afzelii* roots. *Nat. Prod. Res.* 23, 909–915. doi: 10.1080/14786410802497240

Omasits, U., Ahrens, C. H., Müller, S., and Wollscheid, B. (2014). Protter: interactive protein feature visualization and integration with experimental proteomic data. *Bioinformatics* 30, 884–886. doi: 10.1093/bioinformatics/btt607

Poivet, E., Gallot, A., Montagné, N., Glaser, N., Legeai, F., and Jacquin-Joly, E. (2013). A comparison of the olfactory gene repertoires of adults and larvae in the noctuid moth *Spodoptera littoralis*. *PLoS ONE* 8:e60263. doi: 10.1371/journal.pone.0060263

Rukachaisirikul, V., Buadam, S., Sukpondma, Y., Phongpaichit, S., Sakayaroj, J., and Hutadilok-Towatana, N. (2013). Indanone and mellein derivatives from the Garcinia-derived fungus Xylaria sp. PSU-G12. *Phytochem. Lett.* 6, 135–138. doi: 10.1016/j.phytol.2012.11.007

Rützler, M., and Zwiebel, L. (2005). Molecular biology of insect olfaction: recent progress and conceptual models. *J. Comp. Physiol. A* 191, 777–790. doi: 10.1007/s00359-005-0044-y

Salama, H. S., Dimetry, N. Z., and Salem, S. A. (1971). On the host preference and biology of the cotton leaf worm *Spodoptera littoralis* Bois. *J. Appl. Entomol.* 67, 261–266. doi: 10.1111/j.1439-0418.1971.tb02122.x

Sánchez-Gracia, A., Vieira, F. G., and Rozas, J. (2009). Molecular evolution of the major chemosensory gene families in insects. *Heredity* 103, 208–216. doi: 10.1038/hdy.2009.55

Saveer, A. M., Kromann, S., Birgerson, G., Bengtsson, M., Lindblom, T., Balkenius, A., et al. (2012). Floral to green: mating switches moth olfactory coding and preference. Proc. Biol. Sci. 279, 2314–2322. doi: 10.1098/rspb.2011.2710

Smadja, C., and Butlin, R. K. (2009). On the scent of speciation: the chemosensory system and its role in premating isolation. *Heredity* 102, 77–97. doi: 10.1038/hdy.2008.55

Steinwender, B., Thrimawithana, A. H., Crowhurst, R. N., and Newcomb, R. D. (2015). Pheromone receptor evolution in the cryptic leafroller species, *Ctenopseustis obliquana* and *C. herana*. *J. Mol. Evol.* 80, 42–56. doi: 10.1007/s00239-014-9650-z

Stensmyr, M. C., Dekker, T., and Hansson, B. S. (2003). Evolution of the olfactory code in the *Drosophila melanogaster* subgroup. *Proc. R. Soc. B* 270, 2333–2340. doi: 10.1098/rspb.2003.2512

Stranden, M., Liblikas, I., König, W., Almaas, T., Borg-Karlson, A.-K., and Mustaparta, H. (2003). (–)-Germacrene D receptor neurones in three species of heliothine moths: structure-activity relationships. *J. Comp. Physiol. A* 189, 563–577. doi: 10.1007/s00359-003-0434-y

Su, C. Y., Menuz, K., and Carlson, J. R. (2009). Olfactory perception: receptors, cells, and circuits. *Cell* 139, 45–59. doi: 10.1016/j.cell.2009.09.015

Suh, E., Bohbot, J., and Zwiebel, L. J. (2014). Peripheral olfactory signaling in insects. *Curr. Opin. Insect Sci.* 6, 86–92. doi: 10.1016/j.cois.2014.10.006

Syrchina, A., and Semenov, A. (1982). Natural indanones. *Chem. Nat. Comp.* 18, 1–11. doi: 10.1007/BF00581584

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197

Trona, F., Anfora, G., Balkenius, A., Bengtsson, M., Tasin, M., Knight, A., et al. (2013). Neural coding merges sex and habitat chemosensory signals in an insect herbivore. *Proc. R. Soc. B* 280:20130267. doi: 10.1098/rspb.2013.0267

Trona, F., Anfora, G., Bengtsson, M., Witzgall, P., and Ignell, R. (2010). Coding and interaction of sex pheromone and plant volatile signals in the antennal lobe of the codling moth *Cydia pomonella*. *J. Exp. Biol.* 213, 4291–4303. doi: 10.1242/jeb.047365

Witzgall, P., Ansebo, L., Yang, Z., Angeli, G., Sauphanor, B., and Bengtsson, M. (2005). Plant volatiles affect oviposition by codling moths. *Chemoecology* 15, 77–83. doi: 10.1007/s00049-005-0295-7

Witzgall, P., Proffit, M., Rozpedowska, E., Becher, P. G., Andreadis, S., Coracini, M., et al. (2012). "This is not an apple" - yeast mutualism in codling moth. *J. Chem. Ecol.* 38, 949–957. doi: 10.1007/s10886-012-0158-y

Yang, B., Ozaki, K., Ishikawa, Y., and Matsuo, T. (2015). Identification of candidate odorant receptors in asian corn borer *Ostrinia furnacalis*. *PLoS ONE* 10:e0121261. doi: 10.1371/journal.pone.0121261

Zhang, J., Wang, B., Dong, S. L., Cao, D. P., Dong, J. F., Walker, W. B., et al. (2015a). Antennal transcriptome analysis and comparison of chemosensory gene families in two closely related noctuid moths, *Helicoverpa armigera* and *H. assulta*. *PLoS ONE* 10:e0117054. doi: 10.1371/journal.pone.0117054

Zhang, J., Yan, S., Liu, Y., Jacquin-Joly, E., Dong, S., and Wang, G. (2015b). Identification and functional characterization of sex pheromone receptors in the common cutworm (*Spodoptera litura*). *Chem. Senses* 40, 7–16. doi: 10.1093/chemse/bju052

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Positive selection in extra cellular domains in the diversification of *Strigamia maritima* chemoreceptors

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The recent publication of a centipede (*Strigamia maritima*) genome has revealed that most members of the chemosensory gene families of ionotropic (IR) and gustatory (GR) receptors do not have identifiable orthologs in insect species. In other words, the diversity of these chemoreceptors in centipedes appears to have evolved after its split from other arthropod lineages. Here we investigate the role of adaptive evolution in *S. maritima* chemoreceptor diversification using an approach that allows us to discuss functional aspects of such diversification. We applied codon substitution models in a phylogenetic framework to obtain the distribution of selective constraints across the different domains in the IR and GR proteins, and to assess the impact of positive selection in the evolution of these chemoreceptors. We found low selective constraints in most IR and GR duplicates and significant evidence for the presence of positively selected amino acids in two of the four IR, and in six of the GR recent specific expansions. Mapping the sites with high posterior probability of positive selection in protein structure revealed a remarkable uneven distribution of fast-evolving sites across protein domains. Most of these sites are located in extracellular fragments of these receptors, which likely participate in ligand recognition. We hypothesize that adaptive evolution in ligand-binding domains was a major force driving the functional diversification of centipede chemoreceptors.

Keywords: positive selection, functional domains, chemosensory function, gustatory receptor, ionotropic receptor

Introduction

The chemosensory system of arthropods is an interesting subject to study evolution due to its adaptive value. Chemosensation is necessary for finding food, avoiding predators, and locating and choosing mates. The system is composed of proteins encoded by small to medium-sized gene families of two main types: chemosensory (membrane) receptors and ligand-binding proteins (Pelosi et al., 2006; Sánchez-Gracia et al., 2009, 2011; Touhara and Vosshall, 2009). First studied in *Drosophila melanogaster*, three chemoreceptor gene families were identified: Olfactory receptors (OR), Gustatory receptors (GR), and Ionotropic receptors (IR) (Robertson et al., 2003; Benton et al., 2009). With the sequencing of other arthropod genomes, it became evident that the ORs are unique to insects (Peñalva-Arana et al., 2009; Chipman et al., 2014). Members of the GR and IR gene families were, in contrast, found in all arthropod genomes sequenced so far. In fact, the IRs and GRs seem to have an even older origin; they are present in non-arthropod animals, but GRs probably did not always have a chemosensory function

(Croset et al., 2010; Rytz et al., 2013; Saina et al., 2015; Hugh Robertson unpublished). In *D. melanogaster*, olfaction, or the perception of airborne or volatile chemicals, is mediated by the ORs. GRs are implicated in the perception of soluble chemicals and CO_2, while IRs have been implicated in the detection of both soluble and airborne cues (Vosshall and Stocker, 2007; Rytz et al., 2013). Arthropods have a marine ancestry, but there were several independent invasions of land, such as in the Chelicerates, Myriapods, and Insects (Rota-Stabelli et al., 2013). Since the ORs are not present in the Chelicerates and Myriapods (Chipman et al., 2014; Frías-López et al., 2015; Hugh Robertson unpublished results, Julio Rozas unpublished results), an evident question is which genes encode receptors for air-borne cues in these land arthropods.

Here, we begin to investigate this question by analyzing the evolution of the GR and IR families in a myriapod that has recently had its genome sequence published: *Strigamia maritima* (Chipman et al., 2014). This species is found along the coastline of northwestern Europe and has been used as a model system in developmental studies (Kettle et al., 2003; Arthur and Chipman, 2005; Chipman and Akam, 2008; Green and Akam, 2013). Interestingly, it belongs to a centipede Order (Geophilimorphs) that includes animals that have completely lost their eyes, which suggests they rely heavily on other sensory systems. A search for GR and IR orthologs in the *S. maritima* genome identified 76 GRs (13 of which are pseudogenes) and 54 IRs (3 of which are pseudogenes) (Chipman et al., 2014). These numbers are well within the range of gene family members in other arthropod species. In both families, however, only a few 1:1 orthologous relationships were found with other arthropod chemoreceptors (Chipman et al., 2014). Instead, phylogenetic analyses for each chemoreceptor family revealed that all *S. maritima* GR and most IR genes clustered together in a single clade without close relationships to orthologs in other arthropod species. This result suggests that the observed diversity of myriapod chemosensory receptors evolved after this lineage split from its last common ancestor with other arthropods. The only genes with an identifiable candidate ortholog in other arthropods were three IRs (SmarIR25a, SmarIR8a, and SmarIR49) with antennal expression in *D. melanogaster*, which points to a role in olfaction in flies (Chipman et al., 2014). Namely, IR25a is a highly conserved gene in Protostomia and appears to have kept its chemosensory function throughout the evolution of this group (Croset et al., 2010). In fact, this gene is broadly expressed in *Drosophila* olfactory tissues and might represent a common subunit in different IR complexes with a function analogous to that of Orco (Sato et al., 2008; Croset et al., 2010).

To gain insights into the evolution of functional properties of the *S. maritima* GR and IR repertoires, we investigated the selective pressures acting during the diversification of duplications that led to the observed diversity of lineage specific genes. It is generally believed that gene duplications allow relaxed evolution of one or both gene copies for at least some time following the duplication event (Ohno, 1970; Innan and Kondrashov, 2010). Over time, while some copies may accumulate deleterious mutations and eventually cease to be functional, others may instead evolve under positive selection for an acquired beneficial mutation. In the latter case, the copy may be maintained due to its functional differentiation in a process called neofunctionalization (Ohno, 1970). Another, non-exclusive explanation for the lineage specific expansions observed in the chemosensory gene families is the stochastic nature of the gene birth and death process. According to this hypothesis, the maintenance, and loss of duplicated gene copies can be mostly explained by an entirely random process dubbed random genomic drift, which is characterized by only two parameters: gene birth and death rates (Nei, 2007; Nei et al., 2008). Here we make use of state-of-the-art phylogenetic methods that allow for the exploration of genomic data to understand very specific details of gene family functional evolution. We used these methods to test whether positive selection was involved in GR and IR paralog divergence and in this way evaluate the role of selection in the evolution of these gene families in *S. maritima*. This approach permits the identification of amino acid residues likely under positive selection and consequently which gene regions and amino acid sites are under selective pressure for diversification. This study is a fundamental genomics contribution to the functional aspects of the chemosensory receptors of *S. maritima* and lays the ground for further examination of *S. maritima*'s chemosensory system through experimental approaches.

Materials and Methods

Dataset

All gene sequences used in this study were manually curated by us and published in Chipman et al. (2014). In brief, we ran a number of similarity searches on the *S. maritima* genome to identify candidate *S. maritima* GR and IR genes. We then superimposed the results of these searches, available EST information, and the automated gene predictions on the genome to manually annotate each chemosensory gene and obtain the dataset we used in the present study (see details in Chipman et al., 2014; *S. maritima* IR and GR CDS sequences are included in the Supplementary Material).

The methodological approach we used herein to detect signs of positive selection depends on sequence variation and its accuracy is reduced if it is applied to very divergent paralogs. To circumvent this problem, we first identified recent clades based on synonymous divergence in both the GR and the IR gene families within which separate analyses would be carried out (**Figure 1**). For that, we first obtained a multiple alignment of amino acid sequences with MAFFT v. 7 (Katoh and Standley, 2013) using default parameters and used it in a maximum likelihood search to obtain a gene tree using the program RAxML v.8 (Stamatakis, 2006) with the PROTGAMMAWAG model. We were aware that the *S. maritima* GRs and IRs most likely do not represent species-specific chemosensory expansions since we have low phylogenetic coverage in this part of the arthropod tree (the most recent common ancestor of centipede and insects from which we have IR sequences was ∼700 mya); chemoreceptors from other myriapod species are expected to spread out across the *S. maritima* clades. Nevertheless, some of the considered sub-clades (**Figure 1**) are relatively recent and they could in

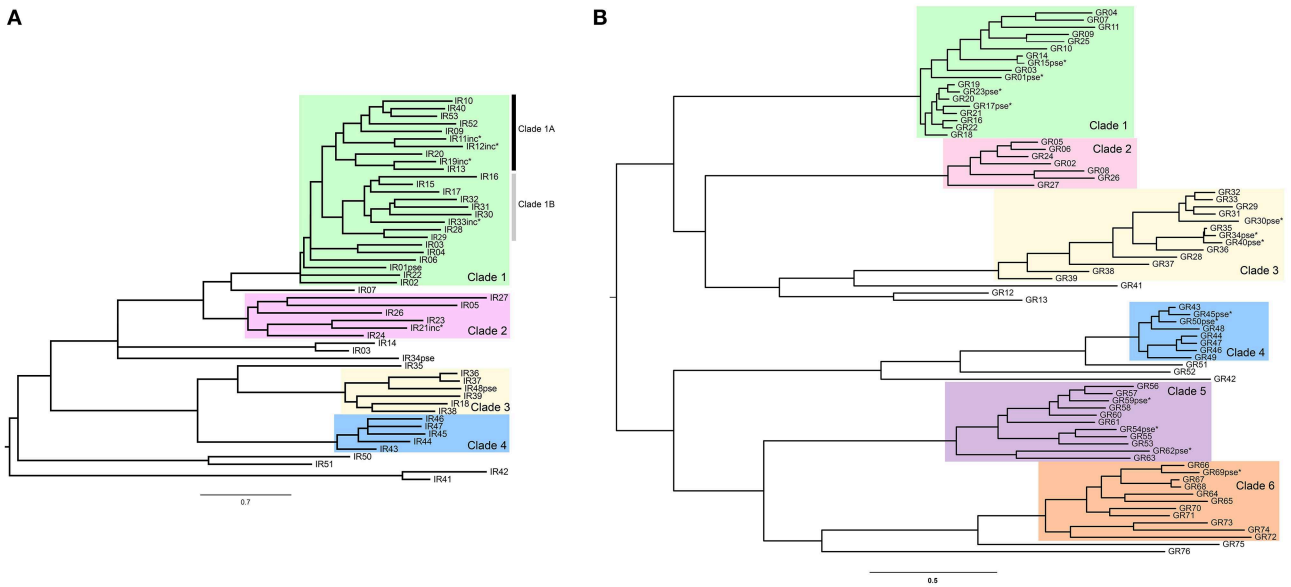

FIGURE 1 | Maximum likelihood phylogenetic trees highlighting (in colored boxes) the Clades with IR and GR genes used in codon substitution model-based analyses. Putative pseudogenes and incomplete copies (indicated with asterisks) were not used for the analysis. **(A)** IR tree. **(B)** GR tree.

fact represent recent paralogous expansions. Both gene families showed expanded clades with more than five recently diverged paralogs (**Figure 1**). In the GR family, six main clades were identified, while in the IR family there were four clades. One IR clade (Clade 1, **Figure 1A**) was further subdivided into two subclades to maximize the number of analyzed positions and the power of the ML analyses. Notice that these clades only include the paralogous genes belonging to the centipede specific expansion. We then obtained separate multiple alignments of the nucleotide sequences of the genes included in each clade with the program MAFFT (Katoh and Standley, 2013) for the statistical analyses. Pseudogenes, i.e., fragments with early stop codons and/or frameshift mutations, were excluded from the analysis since they have inflated mutation rates that are not adaptive.

Statistical Tests for Positive Selection

We used the codon-based substitution models (Nielsen and Yang, 1998; Yang et al., 2000) implemented in *codeml* program (PAML 4.4; Yang, 2007) to estimate non-synonymous to synonymous substitution rate (d_N/d_S) ratios (ω) across paralog sequences. To determine whether there is significant evidence of evolution by positive selection in recently diverged paralogs, we used *codeml* to estimate the goodness of fit of different models to the observed data using a maximum likelihood approach. First, we fit to the data a model with one single average ω (Model M0) in order to obtain the branch lengths to be used as initial values for more complex models. Then we estimated model parameters and the log likelihood (L) of the data under two alternative models: M7, a model with ten classes of sites with beta distributed ω-values in the interval (0–1) (no positively selected sites allowed) and M8, where an extra class of sites (with proportion p_s) constrained to have $\omega_s \geq 1$ is added to the beta model. We compared the L of these two models by using the likelihood ratio test (LRT; $\alpha = 0.05$ after controlling for the false discovery rate—FDR; Benjamini and Hochberg, 1995). As pointed out by Swanson et al. (2003) the M7 vs. M8 comparison may result (in some particular cases) in a high proportion of false positives (significant tests in the absence of positive selection). In fact, the test only indicates if there is a class of sites with a $\omega > 1$ but not if this ratio is significantly greater than 1. Hence, we also used the M8a model, which was proposed as an alternative null hypothesis in which the extra class in M8 is fixed to $\omega = 1$, making a more refined (and conservative) test for the existence of positively selected sites (when compared to model M8). We assumed that the asymptotic null distribution of the LRT statistic is a c^2 (df = 2) for the M8 vs. M7 comparison and a 50:50 mixture of point mass 0 and c^2 (df = 1) for M8 vs. M8a comparison (Self and Liang, 1987; Swanson et al., 2003). All analyses were repeated using different ω starting values to avoid local optima in the maximum likelihood calculation. The Bayes Empirical Bayes (BEB; Yang et al., 2005) analysis under model M8 was used to identify codons under positive selection. Briefly, this method calculates, for each site in the alignment, the posterior probability (PP) of belonging to each of the different omega site classes defined in the model (11 classes in M8). Sites with $PP > 0.5$ of belonging to the class of sites with $\omega > 1$ are candidates to have been under positive selection.

Delimiting Functional Domains

To evaluate whether sites with a probability of being under positive selection have a random or patterned distribution across the different protein domains, we mapped the amino acids corresponding to these codons in the predicted functional domains of the IR and GR receptors. We used different pieces of information to delimit these domains. For IRs, we first

predicted the transmembrane segments in the multiple sequence alignment of each clade using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) with default settings. Then we delimited the pore loop, S1 and S2 ligand binding domains of *S. maritima* IRs using the alignments of the amino acid sequences of *Drosophila* and human iGluRs and *Drosophila* IRs in Benton et al. (2009) as a guide (based on the sequence similarity with some highly conserved residues).

For GRs, the prediction of the transmembrane domains is less clear-cut because not all of the domains are as prominently hydrophobic as most transmembrane proteins such as GPCRs, channels, and transporters, and various transmembrane prediction programs regularly under-predict them and sometimes over-predict them; they consequently also often indicate the incorrect orientation in the membrane (e.g., Benton et al., 2006; Smart et al., 2008; Zhang et al., 2011; Hull et al., 2012). The orientation in the membrane is known for the conserved Odorant Receptor Co-Receptor or Orco protein and other ORs (Benton et al., 2006; Lundin et al., 2007; Smart et al., 2008; Tsitoura et al., 2010; Hull et al., 2012), which are clearly related to the GR family in the insect chemoreceptor superfamily (Robertson et al., 2003), as well as for one GR (Zhang et al., 2011). It is the opposite of the GPCR orientation, with the N-terminus internal to the cell. The transmembrane domains were therefore defined on the basis of a combination of hydrophobic regions highlighted in CLUSTALX (Larkin et al., 2007) alignments and Kyte-Doolittle hydropathy plots (Kyte and Doolittle, 1982).

We tested whether amino acid sites with $PP > 0.5$ of being one of the positive selected sites in the BEB analysis were homogeneously distributed across functional domains. For that, we built a contingency table ($2 \times n$ table, where n is the number of domains defined in each receptor) to compare the relative number of sites with $PP > 0.5$ with the number of sites with $PP < 0.5$ across domains. In this way, the total number of sites of each domain is implicitly taken into account, removing the effect of domain length. We used the Fisher's exact test and FDR (False Discovery Rate—when doing multiple comparisons across clades) to obtain *P*-values.

Results and Discussion

Functional Constraints

The number of genes per clade varied from 5 to 20 in the IRs and the total tree length in number of substitutions per codon varied from 2.59 to 13.24 (**Table 1**). These medium-to-high levels of nucleotide divergence have been found to maximize power and accuracy of the LRT (Anisimova et al., 2001). The ω-values across alignment (*codeml* model M0) never reached 1, being largest ($\omega = 0.579$) for the IR Clade 1A (**Table 1**). The lowest value ($\omega = 0.221$) was observed in Clade 2, which was also the oldest clade as inferred from the longer branch lengths separating its members (**Figure 1A**). Among the GRs, the number of genes per analyzed clade varied from 7 to 14 and the maximum tree length was 7.99 in GR Clade 6 (**Table 1**). The ω-values were in a similar range (0.337–0.646) to that observed in the IR family, although a little higher, on average (**Table 1**). Interestingly, this is in accordance with the GR family having on average the lowest selective constraint among chemosensory families observed in *Drosophila* (Sánchez-Gracia et al., 2009).

TABLE 1 | Characteristics of the codon sequence alignments per clade.

Family	Clade	*N*	*C*	*T*	ω^a
IR	Clade 1	20	267	13.24	0.478
	Clade 1A	7	336	5.09	0.579
	Clade 1B	8	306	6.57	0.511
	Clade 2	5	373	9.02	0.221
	Clade 3	5	482	3.23	0.483
	Clade 4	5	561	2.59	0.441
GR	Clade 1	14	375	6.00	0.479
	Clade 2	7	376	3.91	0.444
	Clade 3	10	382	6.05	0.420
	Clade 4	7	376	2.79	0.646
	Clade 5	8	372	5.59	0.580
	Clade 6	10	345	7.99	0.377

N, number of sequences; C, number of codons; T, total tree length (in number of substitutions per codon).

[a] *Average d_N/d_S over all alignment positions analyzed.*

All the estimated ω-values are relatively high as compared to values found in ortholog comparisons across several gene families in *Drosophila* (Clark et al., 2007). Chemosensory genes, however, may have extraordinarily low functional constraints, with high ω-values even in among-ortholog comparisons as compared to the ω-values obtained for other *Drosophila* gene families (Sánchez-Gracia et al., 2009, 2011). In some *Drosophila* chemosensory ortholog comparisons, ω-values were as high as the ones observed here among paralogs; these were outliers, however, as gene family averages of ω estimates from ortholog comparisons were never higher than 0.25 (Sánchez-Gracia et al., 2011). The relatively high ω-values we observed among *S. maritima* IR and GR paralogs is in accordance with a relaxation of purifying selection as expected in duplicated genes (Innan and Kondrashov, 2010) and observed in chemosensory gene families in other species (Smadja et al., 2009; Almeida et al., 2014). On the other hand, ω-values below one suggest that although selective constraints are relaxed and probably some codons might be under positive selection, most codons are evolving under purifying selection in these genes.

Tests for Positive Selection

The presence of positively selected sites accounting for the increased ω-values observed in the *S. maritima* paralogs comparisons was statistically supported in IR Clade 1 and IR Clade 4, and all six GR clades (**Table 2**). Among the IR clades, the strongest signal of positive selection was in the larger and more recently expanded clade of centipede IRs (Clade 1, LR *P*-values in **Table 2**), both considering the clade as whole and separately for each of the two main sub-clades (Clade 1A and 1B, **Figure 1A**). The range of ω-values of the positively selected sites in IRs (1.2–2.7), estimated under the M8 model, is slightly smaller but similar to those estimated for GRs (1.4–5.5). The proportion of sites estimated to have an $\omega > 1$ ranged from $p_1 = 4.6$–26.7% in IRs and $p_1 = 2.1$–20.8% in GRs, which indicates high levels

TABLE 2 | Results of likelihood ratio tests and parameters estimates under the best-fitting model for each clade.

	Clade	M8 vs. M7 LR (*P*-value)[a]	M8 vs. M8a LR (*P*-value)[a]	Parameter estimates[b]	PSS[c]
IR	Clade 1	26.48 (**5.33×10^{-6}**)	11.78 (**5.98×10^{-4}**)	$p_0 = 0.916$, $p_1 = 0.084$, $\omega_1 = 1.576$	21(3)
	Clade 1A	34.79 (**1.67×10^{-7}**)	17.39 (**6.09×10^{-5}**)	$p_0 = 0.886$, $p_1 = 0.114$, $\omega_1 = 2.279$	27(3)
	Clade 1B	17.89 (**8.28×10^{-4}**)	3.38 (**0.033**)	$p_0 = 0.733$, $p_1 = 0.267$, $\omega_1 = 1.206$	42(1)
	Clade 2	0.004 (0.998)	-	-	not allowed
	Clade 3	2.26 (0.451)	-	-	not allowed
	Clade 4	15.00 (**6.01×10^{-4}**)	3.74 (**0.033**)	$p_0 = 0.954$, $p_1 = 0.046$, $\omega_1 = 2.738$	17(2)
GR	Clade 1	23.25(**1.07×10^{-5}**)	7.68 (**5.58×10^{-3}**)	$p_0 = 0.792$, $p_1 = 0.208$, $\omega_1 = 1.408$	48(2)
	Clade 2	34.59 (**6.16×10^{-8}**)	23.02 (**2.40×10^{-6}**)	$p_0 = 0.922$, $p_1 = 0.078$, $\omega_1 = 2.956$	17(3)
	Clade 3	21.90 (**1.76×10^{-5}**)	17.96 (**2.71×10^{-5}**)	$p_0 = 0.979$, $p_1 = 0.021$, $\omega_1 = 5.493$	8(3)
	Clade 4	46.04 (**3.02×10^{-10}**)	32.65 (**3.30×10^{-8}**)	$p_0 = 0.925$, $p_1 = 0.075$, $\omega_1 = 4.458$	20(8)
	Clade 5	33.58 (**7.66×10^{-8}**)	27.57 (**3.03×10^{-7}**)	$p_0 = 0.854$, $p_1 = 0.146$, $\omega_1 = 2.447$	54(4)
	Clade 6	52.44 (**2.46×10^{-11}**)	34.74 (**2.26×10^{-8}**)	$p_0 = 0.913$, $p_1 = 0.087$, $\omega_1 = 3.258$	25(7)

[a] *LR = Likelihood Ratio ($2\Delta L$). In parentheses are P-values after controlling for FDR (see text for details).*

[b] *Estimated parameters under the M8 model (when significant): p_0 = proportion of sites that follow a beta distribution with 10 ω classes ($0 \leq \omega \geq 1$); p_1 = proportion of sites in the extra class with $\omega \geq 1$.*

[c] *PSS, number of predicted sites under positive selection ($PP > 0.50$). The number of PSS sites with $PP > 0.95$ is shown in parentheses.*

of positive selection acting during the diversification of these receptors. Evidence of positive selection in recently duplicated chemosensory genes has also been found in *Drosophila* using similar site-specific methods (Croset et al., 2010) or using a branch-model approach (Almeida et al., 2014). The application of the latter methods demonstrated that the evidence of positive selection is stronger in the divergence among recent paralogs than in orthologous diversification. In the present work we applied for the first time a model accounting for heterogeneous selective pressure across sites to study the diversification of chemoreceptor paralogs in a non-insect species.

Distribution of Positively Selected Sites

Given the highly significant LRT attesting for the presence of positively selected sites in the analyzed paralogs, we used the BEB analysis to predict the putative location of such sites (Supplementary Tables S1–S10). The BEB analysis, however, had a low performance in pinpointing the specific codon sites affected (i.e., positions with strong posterior probability (*PP*) to belong to the $\omega > 1$ site class). The number of sites with $PP > 0.95$ in IRs was very low (e.g., 3 out of 21 sites with probability to be under positive selection in IR proteins of Clade 1), with no site with $PP > 0.99$ (the sites with $PP > 0.95$ and $PP > 0.7$ belonging to the positively selected class were 9 and 22, respectively). In the GR family, the number of sites with high probability is somewhat higher; the GR Clade 4 had the highest number of sites with $PP > 0.95$ (8), followed by GR Clade 6 (7).

To investigate how selective pressure is distributed across functional elements, we mapped the location of all amino acid sites under positive selection ($PP > 0.5$) in the predicted functional domains of the IR and GR proteins. The structure of the IRs is characterized by an N-terminal domain (N-term), which is highly variable in size, a bipartite ligand-binding domain (LDB), three transmembrane domains (M1–M3), an ion channel domain composed of a pore loop (P-loop) located between M1 and M2 and a short C-terminal domain (C-term) (Croset et al., 2010; Traynelis et al., 2010). The LDB recognizes specific ligands that trigger the opening of the ion channel pore (Armstrong et al., 1998). The structure of the GRs consists of seven transmembrane domains (M1-7), three extracellular short loops (ECL1-3), three intracellular long loops (ICL1-3), an extracellular C-terminus (C-term), and an intracellular N-terminus (N-term) (Clyne et al., 2000). The GRs are evolutionarily related to the ORs, sharing the same basic structure. Although little is known about the specific function of the GRs' domains, some information has been gathered on the ORs' domains that could be extrapolated. For instance, several studies have shown that the outer edge of different transmembrane domains affect ligand specificity (Nichols and Luetje, 2010; Pellegrino et al., 2011; Leary et al., 2012; Hughes et al., 2014).

In a lineage specific diversification of chemosensory genes, such as the observed expansions of IRs and GRs in centipedes, it would be expected that the main domains under positive selection would be the ones affecting ligand recognition/specificity. Positive selection for diversification in these domains would lead to an increase in the number of chemicals able to be sensed by the system. In agreement with this expectation, using a branch-site model, Croset et al. (2010) identified a positively selected site ($PP > 0.95$) in the LBD of a recently duplicated IR of *Drosophila mojavensis*. Here, we found that the vast majority (87%) of the IR residues predicted to be under positive selection (including all sites predicted with $PP > 0.95$) are in the extracellular loops (**Figures 2A–D**, Supplementary Tables S1–S4). In the proteins of IR Clade 1, most of these residues were located in the S1 and S2 domains, while those of the IR Clade 4 preferentially accumulated in the large N-terminal domain. We also inferred a number of positively selected sites in the P-loop domain, suggesting that such structure might also have an important role in the functional diversification of IR paralogs. This pattern is specific to this

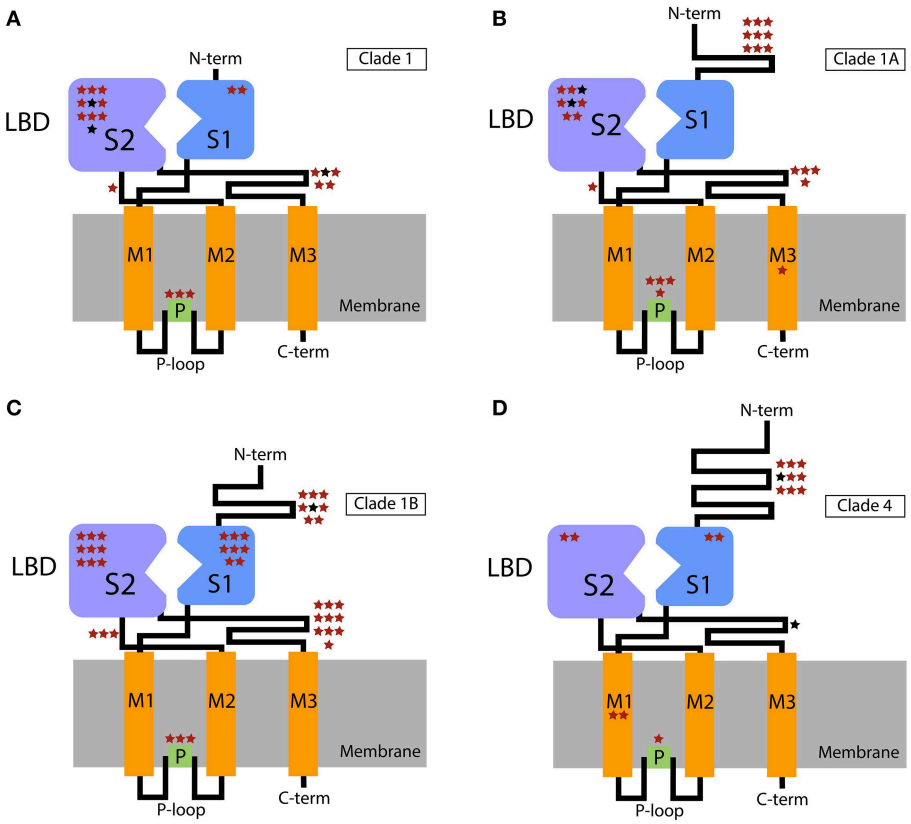

FIGURE 2 | Schematic representation of the IR domains with the extra- and intracellular regions shown above and below the cellular membrane, respectively. Stars show the putative location of positively selected sites across functional domains. The location of these sites within the domains is arbitrary. **(A)** Clade 1; **(B)** Clade 1A; **(C)** Clade 1B; **(D)** Clade 4. Sites with $PP > 0.5$ and $PP > 0.95$ in the BEB analysis are indicated in red and black, respectively. P-loop, ion channel pore loop; M, transmembrane domains; LBD, ligand-binding domain (S1 + S2 lobes); C-term, C-terminal; N-term, N-terminal.

kind of ion channel receptor. Importantly, the accumulation of positively selected sites in specific domains (in this case the LBD and P-loop domains) is significant and not explained by the relative length (in number of amino acids) of these domains within the protein ($P = 1.27 \times 10^{-15}$ or $P = 6.28 \times 10^{-12}$ taking together the sites analyzed for proteins of IR Clade 1 and IR Clade 4, or IR Clade 1A, IR Clade 1B and IR Clade 4, respectively), according to the results of the Fisher's exact test. Considering each clade separately, we found a significant departure from a homogeneous distribution of candidate sites across domains in all cases ($P < 0.006$, after controlling for FDR), except for IR Clade1A ($P = 0.424$). The LBD and the P-loop, therefore, have made a major contribution to the functional diversification of IR paralogs in centipedes.

Similarly, in the GR family the majority of the candidate sites are located in outer parts of the proteins (**Figures 3**, **4**). Overall, the ECL1-3 and the outer sections of the transmembrane domains accumulate more candidate sites than expected given their relative length within the protein ($P = 0.021$). This is very evident for GR Clades 1, 3, 5, and 6 ($P < 0.02$ after controlling for FDR; **Figures 3B,D–F**, Supplementary Tables S5–S10). In these proteins the sites with $PP > 0.95$ also grouped in the ECL1-3 and transmembrane domains ($P = 1.61 \times 10^{-12}$; **Figure 4**), indicating an important functional role of these amino acids. Although there is no functional study focused on GRs, several authors have identified specific amino acids that are involved in ligand specificity of particular ORs. For instance, Nichols and Luetje (2010) found that the outer edge of TM3 of DmOr85b affects ligand specificity; Pellegrino et al. (2011) found that a Val91Ala polymorphism at the outer edge of TM2 of DmOr59b affects ligand-specificity; Leary et al. (2012) found that an Ala148Thr substitution at the outer edge of TM3 in a moth pheromone receptor mediated the ability to detect a new pheromone component; and Hughes et al. (2014) found that mutation of Ala195 on the outer edge of TM4 in *Anopheles gambiae* Or15 greatly affects ligand specificity. Hence, we expect that the positively selected sites we found in GR proteins at positions equivalent to those in the ORs have similar ligand-binding functions.

The present results are remarkable in view of the notable differences in the molecular structure and transmembrane configuration of these two receptor families (GRs have seven transmembrane receptors with an extracellular C-terminal domain, while IRs have an inverted topology with three

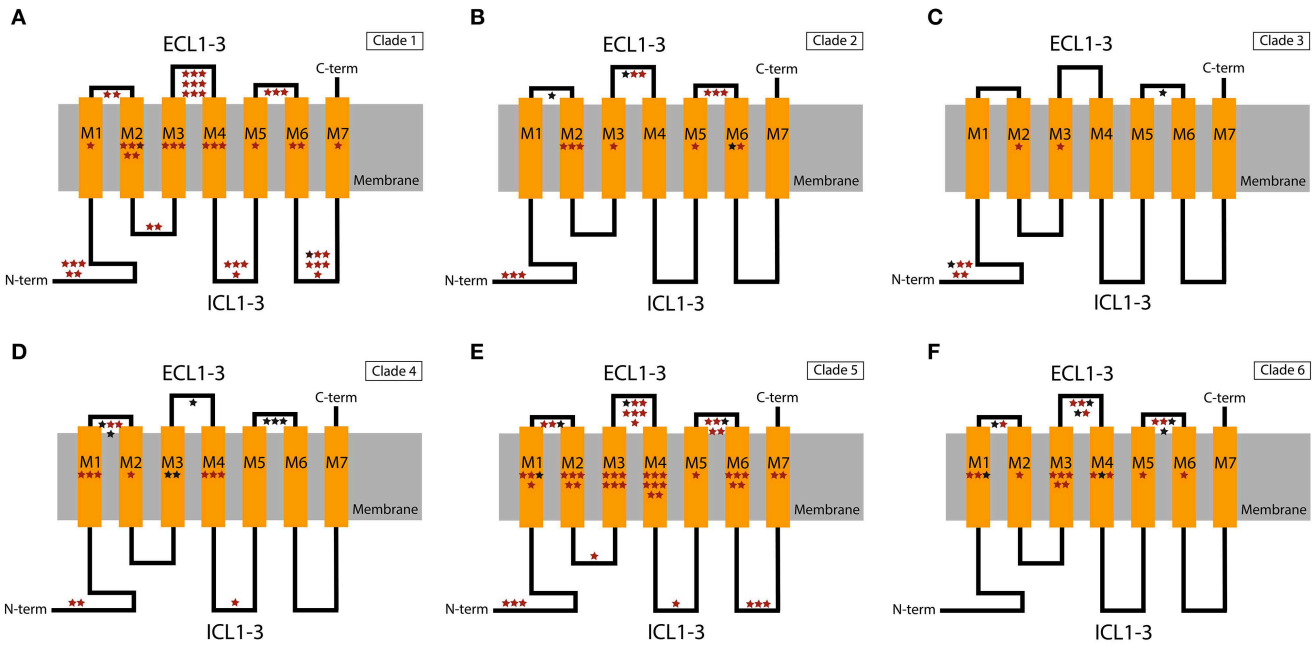

FIGURE 3 | Schematic representation of the GR domains with the extra- and intracellular regions shown above and below the cellular membrane, respectively. Stars show the putative location of positively selected sites in the different functional domains. The location of these sites within the domains is arbitrary. **(A)** Clade 1; **(B)** Clade 2; **(C)** Clade 3; **(D)** Clade 4; **(E)** Clade 5; **(F)** Clade 6. Sites with *PP* > 0.5 and *PP* > 0.95 in the BEB analysis are indicated in red and black, respectively. ECL1-3, extracellular loops 1, 2, and 3; ICL1-3, intracellular loops 1, 2, and 3; C-term, C-terminal; N-term, N-terminal.

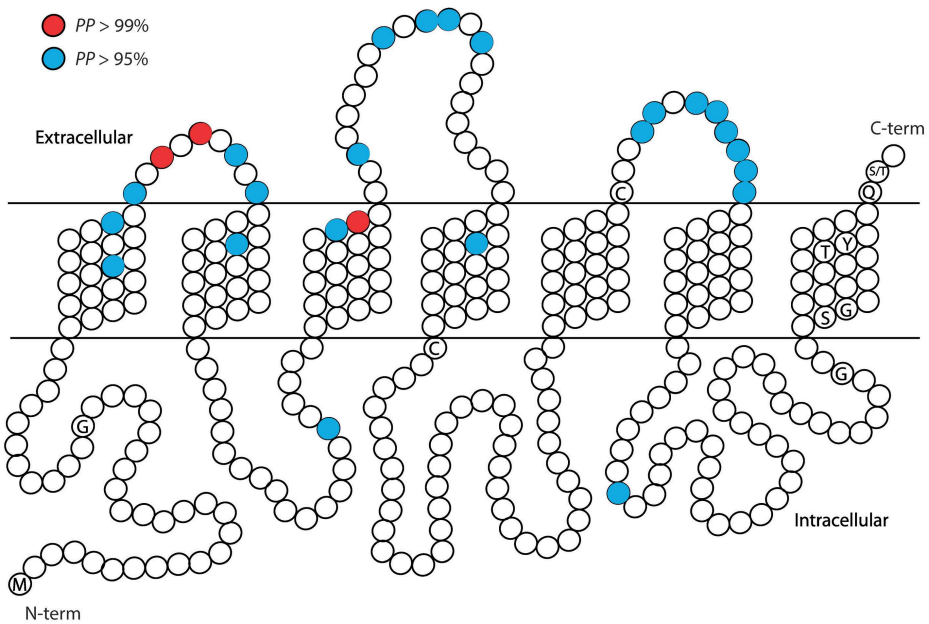

FIGURE 4 | Summary of the approximate positions of the amino acids with high probability to be under positive selection (*PP* > 0.95 in blue; *PP* > 0.99 in red) across the six recent paralogous expansions of *S. maritima* GRs. Each amino acid position is represented with a circle. Characteristic conserved amino acid residues are indicated with the one letter code inside the circle.

transmembrane helices and an intracellular C-terminus). Similar results were obtained with the aphid GRs, in which more than 70% of positively selected sites in paralog comparison were identified in the extracellular region that includes the putative ligand-binding domains (Smadja et al., 2009). Such uneven distribution of positively selected sites across these proteins is reinforcing evidence of selective processes acting on key functional domains. Hence, we can hypothesize that functional diversification of chemosensory receptors would have been largely driven by adaptive changes affecting the ligand-binding specificity and/or sensitivity. Importantly, our results point to specific codons that can, in the future, be targeted in functional experiments to determine ligand specificity and channel characteristics in the GR and IR genes of *S. maritima*.

Very little is known about the molecular apparatus of the chemosensory system of non-insect arthropods. This is a first contribution that explores the potential of molecular evolutionary analyses to contribute to the understanding of functional diversification in chemoreceptors. We found that positive selection has had a role in the evolution of the GR and IR repertoires in *S. maritima* and that adaptive diversification has happened mostly in the ligand-binding interacting domains of these receptors. The next steps in the study of these gene families are the characterization of their expression patterns and of their ligand specificity. The identification of chemosensory genes in other non-insect arthropods would also greatly contribute to our understanding of chemosensation in these less studied organisms.

Author Contributions

FA identified and manually annotated the IR genes. AS did the codon substitution models-based analyses for the IR family and prepared most figures. KW and HR identified and annotated the GR genes and did all the analyses for this gene family. FA was responsible for the primary writing of the manuscript with input from all other authors. All authors participated in the design of the study and interpretation of the data. JR, AS, and FA prepared the final version of the manuscript, which was read and approved by all authors. JR supervised the project.

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Supplementary Material

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References

Almeida, F. C., Sánchez-Gracia, A., Campos, J. L., and Rozas, J. (2014). Family size evolution in *Drosophila* chemosensory gene families: a comparative analysis with a critical appraisal of methods. *Genome Biol. Evol.* 6, 1669–1682. doi: 10.1093/gbe/evu130

Anisimova, M., Bielawski, J. P., and Yang, Z. (2001). Accuracy and power of the likelihood ratio test in detecting adaptive molecular evolution. *Mol. Biol. Evol.* 18, 1585–1592. doi: 10.1093/oxfordjournals.molbev.a003945

Armstrong, N., Sun, Y., Chen, G. Q., and Gouaux, E. (1998). Structure of a glutamate-receptor ligand-binding core in complex with kainate. *Nature* 395, 913–917. doi: 10.1038/27692

Arthur, W., and Chipman, A. D. (2005). The centipede *Strigamia maritima*: what it can tell us about the development and evolution of segmentation. *Bioessays* 27, 653–660. doi: 10.1002/bies.20234

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B Stat. Methodol.* 57, 289–300.

Benton, R., Sachse, S., Michnick, S. W., and Vosshall, L. B. (2006). Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors *in vivo*. *PLoS Biol. Feb* 4:e20. doi: 10.1371/journal.pbio.0040020

Benton, R., Vannice, K. S., Gomez-Diaz, C., and Vosshall, L. B. (2009). Variant ionotropic glutamate receptors as chemosensory receptors in Drosophila. *Cell* 136, 149–162. doi: 10.1016/j.cell.2008.12.001

Chipman, A. D., and Akam, M. (2008). The segmentation cascade in the centipede *Strigamia maritima*: involvement of the Notch pathway and pair-rule gene homologues. *Dev. Biol.* 319, 160–169. doi: 10.1016/j.ydbio.2008.02.038

Chipman, A. D., Ferrier, D. E., Brena, C., Qu, J., Hughes, D. S., Schröder, R., et al. (2014). The first myriapod genome sequence reveals conservative arthropod gene content and genome organisation in the centipede *Strigamia maritima*. *PLoS Biol.* 12:e1002005. doi: 10.1371/journal.pbio.1002005

Clark, A. G., Eisen, M. B., Smith, D. R., Bergman, C. M., Oliver, B., Markow, T. A., et al. (2007). Evolution of genes and genomes on the *Drosophila* phylogeny. *Nature* 450, 203–218. doi: 10.1038/nature06341

Clyne, P. J., Warr, C. G., and Carlson, J. R. (2000). Candidate taste receptors in *Drosophila*. *Science* 287, 1830–1834. doi: 10.1126/science.287.5459.1830

Croset, V., Rytz, R., Cummins, S. F., Budd, A., Brawand, D., Kaessmann, H., et al. (2010). Ancient protostome origin of chemosensory ionotropic glutamate receptors and the evolution of insect taste and olfaction. *PLoS Genet.* 6:e1001064. doi: 10.1371/journal.pgen.1001064

Frías-López, C., Almeida, F. C., Guirao-Rico, S., Vizueta, J., Sánchez-Gracia, A., Arnedo, M. A., et al. (2015). Comparative analysis of tissue specific transcriptomes in the funnel-web spider *Macrothele calpeiana* (*Aranae, Hexathelidae*). *PeerJ* 3:e1064. doi: 10.7717/peerj.1064

Green, J., and Akam, M. (2013). Evolution of the pair rule gene network: insights from a centipede. *Dev. Biol.* 382, 235–245. doi: 10.1016/j.ydbio.2013.06.017

Hughes, D. T., Wang, G., Zwiebel, L. J., and Luetje, C. W. (2014). A determinant of odorant specificity is located at the extracellular loop 2-transmembrane domain 4 interface of an *Anopheles gambiae* odorant receptor subunit. *Chem. Senses* 39, 761–769. doi: 10.1093/chemse/bju048

Hull, J. J., Hoffmann, E. J., Perera, O. P., and Snodgrass, G. L. (2012). Identification of the western tarnished plant bug (*Lygus hesperus*) olfactory co-receptor Orco: expression profile and confirmation of atypical membrane topology. *Arch. Insect. Biochem. Physiol.* 81, 179–198. doi: 10.1002/arch.21042

Innan, H., and Kondrashov, F. (2010). The evolution of gene duplications: classifying and distinguishing between models. *Nat. Rev. Genet.* 11, 97–108. doi: 10.1038/nrg2689

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Kettle, C., Johnstone, J., Jowett, T., Arthur, H., and Arthur, W. (2003). The pattern of segment formation, as revealed by engrailed expression, in a centipede with a variable number of segments. *Evol. Dev.* 5, 198–207. doi: 10.1046/j.1525-142X.2003.03027.x

Kyte, J., and Doolittle, R. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157, 105–132.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947–2948. doi: 10.1093/bioinformatics/btm404

Leary, G. P., Allen, J. E., Bunger, P. L., Luginbill, J. B., Linn, C. E., Macallister, I. E., et al. (2012). Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14081–14086. doi: 10.1073/pnas.1204661109

Lundin, C., Käll, L., Kreher, S. A., Kapp, K., Sonnhammer, E. L., Carlson, J. R., et al. (2007). Membrane topology of the *Drosophila* OR83b odorant receptor. *FEBS Lett.* 581, 5601–5604. doi: 10.1016/j.febslet.2007.11.007

Nei, M. (2007). The new mutation theory of phenotypic evolution. *Proc. Natl. Acad. Sci. U.S.A.* 104, 12235–12242. doi: 10.1073/pnas.0703349104

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. doi: 10.1038/nrg2480

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. doi: 10.1074/jbc.M109.058321

Nielsen, R., and Yang, Z. (1998). Likelihood models for detecting positively selected amino acid sites and applications to the HIV-1 envelope gene. *Genetics* 148, 929–936.

Ohno, S. (1970). *Evolution by Gene Duplication*. New York, NY: Springer-Verlag.

Pellegrino, M., Steinbach, N., Stensmyr, M. C., Hansson, B. S., and Vosshall, L. B. (2011). A natural polymorphism alters odour and DEET sensitivity in an insect odorant receptor. *Nature* 478, 511–514. doi: 10.1038/nature10438

Pelosi, P., Zhou, J.-J., Ban, L. P., and Calvello, M. (2006). Soluble proteins in insect chemical communication. *Cell Mol. Life Sci.* 63, 1658–1676. doi: 10.1007/s00018-005-5607-0

Peñalva-Arana, D. C., Lynch, M., and Robertson, H. M. (2009). The chemoreceptor genes of the waterflea *Daphnia pulex*: many GRs but no ORs. *BMC Evol. Biol.* 9:79. doi: 10.1186/1471-2148-9-79

Robertson, H. M., Warr, C. G., and Carlson, J. R. (2003). Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 100(Suppl. 2), 14537–14542. doi: 10.1073/pnas.2335847100

Rota-Stabelli, O., Daley, A. C., and Pisani, D. (2013). Molecular timetrees reveal a Cambrian colonization of land and a new scenario for Ecdysozoan evolution. *Curr. Biol.* 23, 392–398. doi: 10.1016/j.cub.2013.01.026

Rytz, R., Croset, V., and Benton, R. (2013). Ionotropic Receptors (IRs): chemosensory ionotropic glutamate receptors in *Drosophila* and beyond. *Insect Biochem. Mol. Biol.* 43, 888–897. doi: 10.1016/j.ibmb.2013.02.007

Saina, M., Busengdal, V., Sinigaglia, C., Petrone, L., Oliveri, P., Rentzsch, F., et al. (2015). A cnidarian homologue of an insect gustatory receptor functions in developmental body patterning. *Nat. Commun.* 6, 1–12. doi: 10.1038/ncomms7243

Sánchez-Gracia, A., Vieira, F. G., Almeida, F. C., and Rozas, J. (2011). *Comparative Genomics of the Major Chemosensory Gene Families in Arthropods*. Chichester: eLS, John Wiley & Sons, Ltd.

Sánchez-Gracia, A., Vieira, F. G., and Rozas, J. (2009). Molecular evolution of the major chemosensory gene families in insects. *Heredity* 103, 208–216. doi: 10.1038/hdy.2009.55

Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L. B., and Touhara, K. (2008). Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature* 452, 1002–1006. doi: 10.1038/nature06850

Self, S. G., and Liang, K.-Y. (1987). Asymptotic properties of maximum likelihood estimators and likelihood ratio tests under nonstandard conditions. *J. Am. Stat. Assoc.* 82, 605–610. doi: 10.1080/01621459.1987.10478472

Smadja, C., Shi, P., Butlin, R. K., and Robertson, H. M. (2009). Large gene family expansions and adaptive evolution for odorant and gustatory receptors in the pea aphid, *Acyrthosiphon pisum*. *Mol. Biol. Evol.* 26, 2073–2086. doi: 10.1093/molbev/msp116

Smart, R., Kiely, A., Beale, M., Vargas, E., Carraher, C., Kralicek, A. V., et al. (2008). *Drosophila* odorant receptors are novel seven transmembrane domain proteins that can signal independently of heterotrimeric G proteins. *Insect. Biochem. Mol. Biol.* 38, 770–780. doi: 10.1016/j.ibmb.2008.05.002

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22, 2688–2690. doi: 10.1093/bioinformatics/btl446

Swanson, W. J., Nielsen, R., and Yang, Q. (2003). Pervasive adaptive evolution in mammalian fertilization proteins. *Mol. Biol. Evol.* 20, 18–20. doi: 10.1093/oxfordjournals.molbev.a004233

Touhara, K., and Vosshall, L. B. (2009). Sensing odorants and pheromones with chemosensory receptors. *Ann. Rev. Physiol.* 71, 307–332. doi: 10.1146/annurev.physiol.010908.163209

Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Ogden, K. K., et al. (2010). Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol. Rev.* 62, 405–496. doi: 10.1124/pr.109.002451

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. doi: 10.1371/journal.pone.0015428

Vosshall, L. B., and Stocker, R. F. (2007). Molecular architecture of smell and taste in *Drosophila*. *Annu. Rev. Neurosci.* 30, 505–533. doi: 10.1146/annurev.neuro.30.051606.094306

Yang, Z. (2007). PAML 4: phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* 24, 1586–1591. doi: 10.1093/molbev/msm088

Yang, Z., Nielsen, R., Goldman, N., and Pedersen, A. M. K. (2000). Codon-substitution models for heterogeneous selection pressure at amino acid sites. *Genetics* 155, 431–449.

Yang, Z., Wong, W. S. W., and Nielsen, R. (2005). Bayes empirical Bayes inference of amino acid sites under positive selection. *Mol. Biol. Evol.* 22, 1107–1118. doi: 10.1093/molbev/msi097

Zhang, H. J., Anderson, A. R., Trowell, S. C., Luo, A. R., Xiang, Z. H., and Xia, Q. Y. (2011). Topological and functional characterization of an insect gustatory receptor. *PLoS ONE* 6:e24111. doi: 10.1371/journal.pone.0024111

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Moth pheromone receptors: gene sequences, function, and evolution

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The detection of female-released species-specific sex pheromones in moths is mediated by the pheromone receptors that are expressed in the sensory neurons in the olfactory sensilla of conspecific male antennae. Since the pioneering studies on the tobacco budworm *Heliothis virescens* and the silkworm *Bombyx mori* a decade ago, genes encoding pheromone receptors have been identified from a number of moth species. Pheromone receptor genes constitute a specialized olfactory receptor subfamily that shares sequence homology. In most cases the pheromone receptor genes are more abundantly expressed in male antennae, and the expression is confined to the neurons in the long sensilla trichodea, which are responsible for pheromone sensing. Both highly specific and more broadly tuned pheromone receptors have been described in various moth species. We review the advances in moth pheromone receptor studies over the past decade, including the methods used in receptor gene isolation and functional characterization, the different ligand profiles of the identified receptors, and the evolution of this multigene family.

Keywords: pheromone receptor, lepidoptera, cloning, functional characterization, evolution

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Introduction

Mate-finding behavior, mediated by species-specific sex pheromones, is important in mate recognition in moths. Moth sex pheromones are normally released by adult females during "calling" behavior and tracked by the conspecific males over a long distance. Based on their chemical properties, moth sex pheromones are classified into two major types, Type I sex pheromones comprising C_{10}-C_{18} straight chain fatty alcohols and corresponding acetates and aldehydes, and Type II sex pheromones including long-chain polyunsaturated hydrocarbons and the corresponding epoxides (Millar, 2000; Ando et al., 2004).

The reception of these chemical signals is conducted by specialized pheromone receptors (PRs) expressed in specific olfactory sensory neurons (OSNs) in antennal sensilla. As members of the insect olfactory receptor (OR) family, PRs possess a seven-transmembrane structure and form heteromeric ligand-gated non-selective ion channels with the olfactory co-receptor Orco (Sato et al., 2008). The pheromones are solubilized and transported by pheromone binding proteins (PBPs) through the lymph around the dendrite of the OSNs, and activate the PR/Orco complex (Vogt, 2005). In *Drosophila*, the presence of sensory neuron membrane proteins (SNMPs) is required for proper pheromone-evoked response (Benton et al., 2007). Recent studies indicated that in moth pheromone detection system, SNMPs might contribute to the sensitivity (Pregitzer et al., 2014), or rapid activation and termination of pheromone-induced activity (Li et al., 2014).

Following the pioneering studies on odorant receptors in the vinegar fly, *Drosophila melanogaster* (Clyne et al., 1999; Vosshall et al., 1999), moth PR genes were initially discovered

from two intensively studied species, the tobacco budworm *Heliothis virescens* and the silkworm *Bombyx mori* (Krieger et al., 2004, 2005; Sakurai et al., 2004; Nakagawa et al., 2005; Große-Wilde et al., 2007). Since then, a number of PR genes have been identified from various moth species. In this review, we summarize the progress to date in the isolation and functional characterization of moth PRs, to enable a discussion on the evolution of PR function.

Moth PR Gene Sequences and Expression Pattern

In *H. virescens*, the genomic database was BLAST analyzed with candidate chemosensory receptor genes from *D. melanogaster* and the malaria mosquito *Anopheles gambiae*, combined with screening of antennal cDNA libraries with specific probes (Krieger et al., 2004). In *B. mori*, different cloning strategies were used in two independent studies. BmorOR1 was identified by differential screening of a male antennal cDNA library (Sakurai et al., 2004), whereas more candidate PR genes were identified by the method used in *H. virescens* (Krieger et al., 2005). The sequence homology found in PRs from these two species made it possible to explore new PR genes using degenerate PCR. This approach turned out to be an efficient strategy in various moth species, including the diamondback moth *Plutella xylostella*, the armyworm moth *Mythimna separata*, and the cucumber moth *Diaphania indica* (Mitsuno et al., 2008), the cotton bollworm *Helicoverpa armigera* and the tobacco budworm *Helicoverpa assulta* (Zhang, 2010; Zhang et al., 2010), the European corn borer *Ostrinia nubilalis* and related *Ostrinia* species (Miura et al., 2010; Wanner et al., 2010), the navel orangeworm *Amyelois transitella* (Xu et al., 2012), the beet armyworm *Spodoptera exigua* (Liu et al., 2013a), and the turnip moth *Agrotis segetum* (Zhang and Löfstedt, 2013). The identified PRs cluster in a single lineage, forming a specialized subfamily of olfactory receptors.

The alignment of hitherto known moth PR sequences shows a relatively conserved C-terminal region that contains three highly conserved motifs (**Figure 1A**). Motif 1 has a signature sequence L-(L/M)-(L/V)-(E/Q)-C-(S/T/A); motif 2 contains the signature sequences (Q/G/T)-(Q/E/L)-L-(I/V)-(Q/L/E) and P-W-(E/Q/D); and motif 3 contains the signature sequence (I/V)-(L/I)-(K/R)-(T/A)-(S/T). These motifs provide useful sites for designing degenerate primers to isolate new PR genes. From the functional perspective, the significance of these motifs has not been fully investigated. Previous studies on BmorOR1 in silkworm showed that site-directed mutagenesis of the residue E in the signature sequence L-(L/M)-(L/V)-(E/Q)-C-(S/T/A) or P-W-(E/Q/D) caused functional alterations in the odor-evoked cation channel activity, indicating an essential role of the residues in keeping the PR/Orco complex channel activity (Nakagawa et al., 2012). Further mutagenesis studies will help to define the roles of the other residues in these motifs.

In recent years, RNA sequencing of moth antennal transcriptomes has become a powerful alternative to degenerate PCR when exploring the repertoire of genes coding for olfactory receptors (Montagné et al., 2015). For example, 2 out of 47 ORs of the tobacco hornworm *Manduca sexta*, 5 out of 43 ORs in the codling moth *Cydia pomonella*, and 4 out of 47 ORs in the cotton leafworm *Spodopetra littoralis* were found belonging to the PR subfamily based on the respective transcriptome data (Große-Wilde et al., 2011; Bengtsson et al., 2012; Poivet et al., 2013).

In addition, the expression levels of PR genes may provide clues to receptor function, which can be assessed by *in situ* hybridization and quantitative PCR (Krieger et al., 2005; Wanner et al., 2010; Zhang et al., 2010), or directly from the RNA-seq data. The latter makes it more convenient to compare the expression levels of many target genes in different tissues. In general, the expression level of PR genes is higher in male antennae than in female antennae, and the expression is confined to neurons located in the long sensilla trichodea (Krieger et al., 2005), which are known to be responsive to moth sex pheromones (Schneider, 1974).

Functional Assays of Moth PRs

Different heterologous expression systems have been used to characterize moth PR gene function during the past decade (**Table 1**). The first moth PR, BmorOR1 was deorphanized from *B. mori* using the *Xenopus* oocyte expression system (Sakurai et al., 2004; Nakagawa et al., 2005), which, since then has been most commonly used in moth PR studies (**Table 1** and references therein). In short, the complementary RNAs (cRNAs) of a candidate PR gene and *Orco* gene are co-injected into the oocytes of the African clawed frog, *Xenopus laevis*, where the target receptors are efficiently and faithfully translated, assembled and inserted into the plasma membrane. The oocytes are subsequently incubated and perfused with respective pheromone compounds diluted in buffers. During the perfusion the stimulated inward currents conferred by the PR/Orco heteromeric complex are recorded under the two-electrode voltage clamp (TEVC) at a certain holding potential. The PR ligand profiles obtained from this system agree well with the properties of the olfactory neurons identified by *in vivo* electrophysiological studies, which makes it possible to hypothetically assign the PR genes to corresponding neurons in the sensilla (Miura et al., 2010; Wang et al., 2011; Zhang and Löfstedt, 2013).

Another *in vitro* gene expression system using human embryonic kidney 293 (HEK293) cells was also applied in moth PR functional assays, in which the PRs and G_α proteins are co-expressed in the cells (Große-Wilde et al., 2006, 2007; Forstner et al., 2009), because PRs were previously assumed to be canonical G protein-coupled receptors (GPCR). The coupling of these exogenous proteins elicits an increase in the level of intracellular Ca^{2+} upon pheromone stimulation, which can be monitored by calcium imaging. To improve the response specificity of the transfected HEK293 cells, the matching PBPs were required in above studies. Recently, a functional assay using modified HEK293 cell lines co-expressing PRs with Orco instead of G_α proteins, but in the absence of PBPs was reported (Steinwender et al., 2015), following a previously described protocol for OR study (Corcoran et al., 2014).

The *Drosophila* ¨empty neuron¨ has been employed as an *in vivo* heterologous expression system in moth PR functional assays. Firstly, the flies are genetically modified by replacing

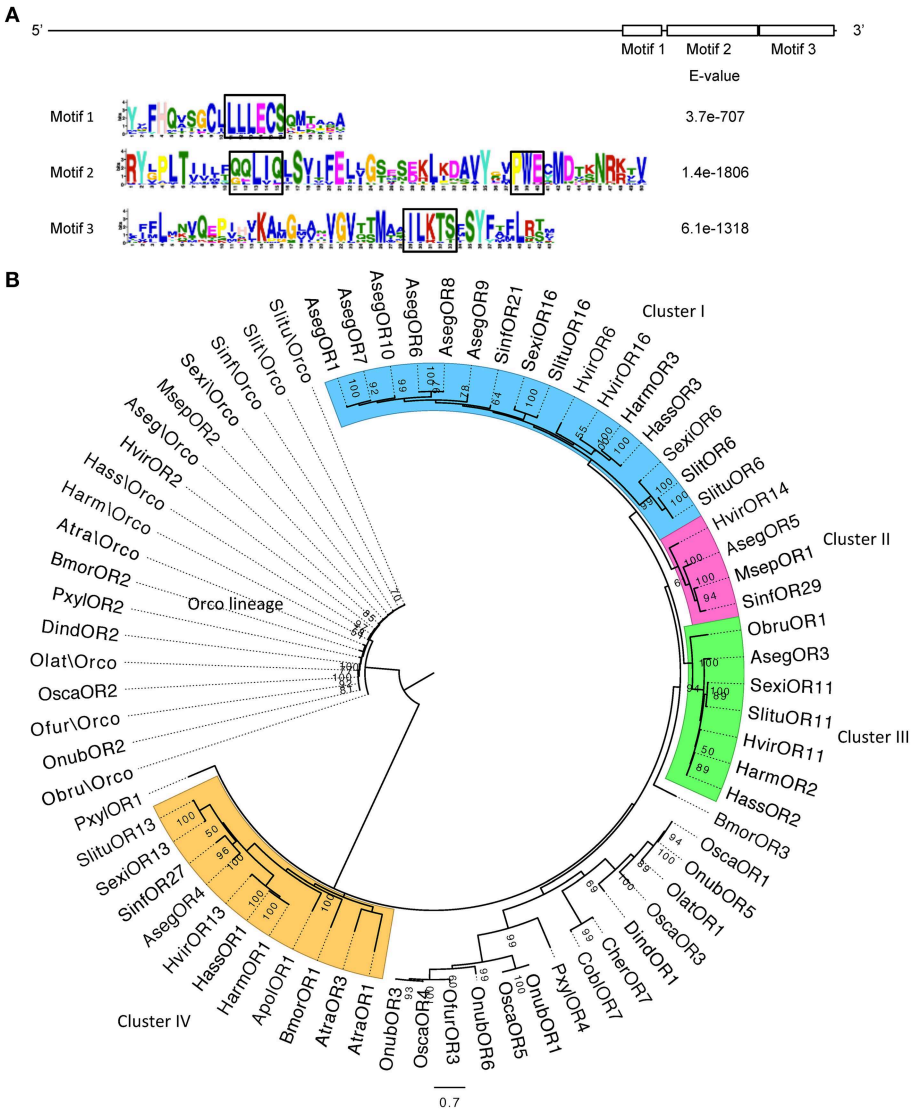

FIGURE 1 | The motif sequences and phylogeny of moth pheromone receptor genes. (A) The upper bar indicates the location of the three motifs on the PR sequences. The lower shows the sequences of the three motifs and respective *E*-values. The signature sequences in the motifs are boxed in black. **(B)** The evolutionary history was inferred with MEGA6 by using the Maximum Likelihood method based on the LG model (Le and Gascuel, 2008; Tamura et al., 2013). The tree with the highest log likelihood (−22599.7) is shown. Support values above 50% are labeled next to the branches, which were derived from 100 bootstrap replicates. Initial tree for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 2.6982)]. The tree was rooted with the Orco lineage. Color coding indicates the four different orthologous clusters.

an endogenous OR gene with a candidate moth PR gene in corresponding *Drosophila* OR-expressing neurons. The antennae of the flies are then stimulated by moth pheromone compounds and the evoked neuronal responses are recorded by single-sensillum recording. The ab3A neurons that host the endogenous *Drosophila DmelOr22a* gene were initially used to express *B. mori* PRs (Syed et al., 2006). However, the T1 neurons that host the *DmelOr67d* gene and respond to the *Drosophila* pheromone cis-vaccenyl acetate (Ha and Smith, 2006) were later found to functionally express moth PR genes more efficiently (Kurtovic et al., 2007; Syed et al., 2010; Montagné et al., 2012). A likely explanation is that the T1 neurons are equipped with necessary components such as SNMP1, which is required for the sensing of sex pheromones in *Drosophila* (Benton et al., 2007).

More recently, a cell-free expression system involving *in situ* protein synthesis has been reported (Hamada et al., 2014). In this study BmorOR1 was co-expressed with Bmor\Orco in giant vesicles and excited in the presence of the ligand bombykol (10*E*,12*Z*)-hexadecadienol, as shown by patch-clamp recording.

To what extent the different assays give similar results is currently not known, when it comes to specificity and sensitivity, but the bulk of available data (**Table 1**) have been collected

using the *Xenopus* oocyte expression system as mentioned above.

Ligand Profiles of Moth PRs

The Specific PRs

A number of PRs are specifically responsive to a single pheromone compound, which in most cases is the major pheromone component for the species in question. The specificity of these PRs confer on them the ability to distinguish compounds sharing very similar chemical structures, including: (1) analogs with different fatty chain lengths, e.g., AsegOR9, AsegOR4, and AsegOR5 in *A. segetum*, which are specifically tuned to the pheromone components (5*Z*)-decenyl, (7*Z*)-dodecenyl, and (9*Z*)-tetradecenyl acetates, respectively (Zhang and Löfstedt, 2013); (2) compounds with the same molecular skeletons but different oxygen-containing functional groups, e.g., BmorOR1 and BmorOR3 in *B. mori* specifically tuned to the sex pheromone components bombykol and its oxidized form bombykal (10*E*,12*Z*)-hexadecadienal, respectively (Nakagawa et al., 2005); (3) stereoisomeric pheromone compounds with different geometry and/or position of the double bond(s), e.g., OnubOR6 in the European corn borer *O. nubilalis Z* strain tuned to (11*Z*)-tetradecenyl acetate, but not to (11*E*)-tetradecenyl acetate (Wanner et al., 2010), OscarOR4 in *O. scapulalis* tuned to (11*E*)-tetradecenyl acetate rather than (12*E*)-tetradecenyl acetate (Miura et al., 2010), and SlitOR6 in *S. littoralis* tuned to (9*Z*,12*E*)-tetradecadienyl acetate, but not to (9*Z*,11*E*)-tetradecadienyl acetate (Montagné et al., 2012).

The Broadly Tuned PRs

In addition to the above-mentioned specific receptors that are tuned to the major pheromone components in respective species, some PRs have broader response spectra. For example, OscaOR3 from *O. scapulalis* responds not only to the conspecific pheromone components (11*E*)- and (11*Z*)-tetradecenyl acetates, but also to those from closely related species, such as (9*Z*)-, (12*E*)-, and (12*Z*)-tetradecenyl acetates (Miura et al., 2010); OnubOR1, OnubOR3, and OnubOR5 from *O. nubilalis* also respond to all the five tetradecenyl acetate isomers mentioned above (Wanner et al., 2010); and similarly, SexiOR16 from *S. exigua* shows broad activity to multiple sex pheromone components (Liu et al., 2013a).

PR Responses to Behavioral Antagonists

Behavioral antagonism mediated by pheromone-like compounds may provide a mechanism for pheromone specificity and prevent cross-attraction between sympatric species and hence reproductive isolation. These compounds can be used as pheromone components in one species, but have antagonistic effects in sibling species (Linn and Roelofs, 1995; Cardé and Haynes, 2004; Linn et al., 2007). The receptors for the behavioral antagonists are also found in the PR subfamily. In *H. virescens*, HvirOR16 and HvirOR14 are specifically responsive to the behavioral antagonists, (11*Z*)-hexadecenol and (11*Z*)-hexadecenyl acetate, respectively (Wang et al., 2011). In some other species, however, the broadly tuned receptors may respond to both their own pheromone compounds and the interspecific behavioral antagonists. For example, the above mentioned OscaOR3 in *O. scapulalis* responds not only to the conspecific pheromone components (11*E*)- and (11*Z*)-tetradecenyl acetates, but also to (9*Z*)-tetradecenyl acetate, a behavioral antagonist in *O. scapulalis* but pheromone component in the closely related species *O. zaguliaevi* and *O. zealis* (Miura et al., 2010). In *S. litura*, in addition to the modest responses to three conspecific sex pheromone components and an analog, SlituOR16 showed the strongest response to (9*Z*)-tetradecenol, a behavioral antagonist in *S. litura*, but a sex pheromone component in *S. exigua* (Zhang et al., 2015b). In *A. segetum*, AsegOR1 responds to both the behavioral antagonist (8*Z*)-dodecenyl acetate and the sex pheromone components (5*Z*)-decenyl and (7*Z*)-dodecenyl acetates; similarly, AsegOR6 responds to both (5*Z*)-decenol, another behavioral antagonist, and the pheromone compound (5*Z*)-decenyl acetate (Zhang and Löfstedt, 2013). The fact that a receptor can respond to both a behavioral agonist and an antagonist might simply because these compounds share similar chemical structures. However, when both agonists and antagonists are perceived, the behavioral outcome might be an olfactory antagonistic balance (Baker, 2008) that depends on the glomerular projection of OSNs and the integration of the information from different receptors in the central nervous system (CNS).

The "Ligand Unknown" Receptors

Among all the moth PRs investigated to date, there is a cluster of orthologous PRs, for which the ligands remain unknown (see Cluster III in **Figure 1B**). The ratio of nonsynonymous to synonymous substitutions (dN/dS value) in this cluster is considerably lower than the other clades, indicating strong purifying selection on the whole cluster, and possibly a conserved function for these receptors (Zhang et al., 2015a). Previous hypotheses of the function of these receptors focused on structurally related pheromone compounds, behavioral antagonists or the degradation products of the major sex pheromone component (Baker, 2009; Krieger et al., 2009). However, these assumptions have not yet received any support from functional analyses. Most recently, our study on pheromone reception in the winter moth, *Operophtera brumata* (Geometridae) has shown that the receptor ObruOR1 in this ligand-unknown cluster is specifically tuned to a tetraene (1,3*Z*,6*Z*,9*Z*)-nonadecatetraene, the single component sex pheromone of this species (Roelofs et al., 1982). Similarly, our subsequent functional characterization of another member of Cluster III, AsegOR3 from the noctuid moth *A. segetum* showed the strongest response to a triene, in this case (3*Z*,6*Z*,9*Z*)-tricosatriene (Zhang et al., 2015a). These results suggest that members in this cluster may all respond to Type II polyene pheromones.

The Evolution of Moth PRs

As mentioned above, the co-existence of specific and more broadly tuned PRs in moths might be a common phenomenon. The highly specific PRs play essential roles in the accurate perception of conspecific pheromones in the presence of structurally similar compounds in the surroundings, ensuring

TABLE 1 | Functionally identified PR genes in lepidopteran species.

Species	Family	Heterologous expression[a]	Genes[b]	Ligands	References
Bombyx mori	Bombycidae	Oocytes with G protein	BmorOR1	E10,Z12-16:OH**	Sakurai et al., 2004
		Oocytes	BmorOR1	E10,Z12-16:OH**	Nakagawa et al., 2005
			BmorOR3	E10,Z12-16:Ald	
		HEK293 with G protein	BmorOR1	E10,Z12-16:OH** (in the presence of PBP)	Große-Wilde et al., 2006
			BmorOR3	E10,Z12-16:Ald	
		DmelOr22a empty neuron	BmorOR1	E10,Z12-16:OH**	Syed et al., 2006
		DmelOr67d empty neuron	BmorOR1	E10,Z12-16:OH**	Syed et al., 2010
		Cell-free (giant vesicles)	BmorOR1	E10,Z12-16:OH**	Hamada et al., 2014
Heliothis virescens	Noctuidae	HEK293 with G protein	HvirOR13	Z11-16:Ald** (in the presence of PBP2)	Große-Wilde et al., 2007
			HvirOR14	Z11-16:OAc, Z11-16:Ald, Z9-16:Ald, Z9-14:Ald*	
			HvirOR16	Z9-14:Ald, Z11-16:OH	
		Oocytes	HvirOR6	Z9-14:Ald*	Wang et al., 2011
			HvirOR11	–	
			HvirOR13	Z11-16:Ald**	
			HvirOR14	Z11-16:OAc[c]	
			HvirOR15	–	
			HvirOR16	Z11-16:OH[c]	
Plutella xylostella	Plutellidae	Oocytes	PxylOR1	Z11-16:Ald**	Mitsuno et al., 2008
		Oocytes	PxylOR4	Z9,E12-14:OAc, Z9-14:OAc	Sun et al., 2013
Mythimna separata	Noctuidae	Oocytes	MsepOR1	Z11-16:OAc**	Mitsuno et al., 2008
Diaphania indica	Crambidae	Oocytes	DindOR1	E11-16:Ald**	Mitsuno et al., 2008
Antheraea polyphemus	Saturniidae	HEK293 with G protein	ApolOR1	E6,Z11-16:Ald* (only specific at low concentration, in the presence of PBP2)	Forstner et al., 2009
Ostrinia latipennis	Crambidae	Oocytes	OlatOR1	E11-14:OH**	Miura et al., 2009
Ostrinia scapulalis (E type)	Crambidae	Oocytes	OscaOR1	E11-14:OH	Miura et al., 2009
		Oocytes	OscaOR3	Broadly tuned to Z11-14:OAc*, E11-14:OAc**, Z12-14:OAc, E12-!4:OAc, and Z9-14:OAc[c]	Miura et al., 2010
			OscaOR4	E11-14:OAc	
			OscaOR5	Marginal responses to a few pheromone components	
			OscaOR6	–	
			OscaOR7	–	
			OscaOR8	–	
Ostrinia nubilalis (Z races)	Crambidae	Oocytes	OnubOR1	Broadly tuned to Z11-14:OAc**, E11-14:OAc*, Z12-14:OAc, E12-14:OAc and Z9-14:OAc[c], more sensitive to E12-!4:OAc	Wanner et al., 2010
			OnubOR3	Broadly tuned to Z11-14:OAc, E11-14:OAc, Z12-14:OAc, E12-!4:OAc and Z9-14:OAc	
			OnubOR4	–	
			OnubOR5	Broadly tuned to Z11-14:OAc, E11-14:OAc, Z12-14:OAc, E12-!4:OAc and Z9-14:OAc	

(Continued)

TABLE 1 | Continued

Species	Family	Heterologous expression[a]	Genes[b]	Ligands	References
			OnubOR6	Z11-14:OAc	
Ostrinia furnacalis	Crambidae	Oocytes	OfurOR3	Preferentially responsive to E12-14:OAc** and Z12-14:OAc**	Leary et al., 2012
Helicoverpa armigera	Noctuidae	Oocytes	HarmOR1	Z11-16:Ald**	Zhang, 2010
			HarmOR2	–	
			HarmOR3	Z11-16:OH[C], Z9-14:Ald	
		Oocytes	HarmOR13	Z11-16:Ald**	Liu et al., 2013b
			HarmOR6	Z9-14:Ald, Z9-16:Ald*	
			HarmOR16	Z11-16:OH, Z9-14:Ald	
			HarmOR11	–	
			HarmOR14	–	
			HarmOR15	–	
Helicoverpa assulta	Noctuidae	Oocytes	HassOR1	Z11-16:Ald*	Zhang, 2010
			HassOR2	–	
			HassOR3	Z9-14:Ald, Z11-16:OH	
		Sf9	HassOR13	Z11-16:Ald*	Xu et al., 2015
Amyelois transitella	Pyralidae	Oocytes	AtraOR1	Z11,Z13-16:Ald**	Xu et al., 2012
			AtraOR3	Z11-16:Ald, Z9,Z11-14OFor	
Spodoptera littoralis	Noctuidae	*DmelOr67d* empty neuron	SlitOR6	Z9,E12-14:OAc*	Montagné et al., 2012
Agrotis segetum	Noctuidae	Oocytes	AsegOR1	Z5-10:OAc, Z7-12:OAc, Z8-12:OAc[C]	Zhang and Löfstedt, 2013
			AsegOR3	Low sensitivity to all the tested compounds	
			AsegOR4	Z7-12:OAc**	
			AsegOR5	Z9-14:OAc*	
			AsegOR6	Z5-10:OH[C], Z5-10:OAc	
			AsegOR7	Similar to AsegOR1, lower sensitivity	
			AsegOR8	Similar to AsegOR6, lower sensitivity	
			AsegOR9	Z5-10:OAc*	
			AsegOR10	Minor responses to Z9-14:OAc, Z5-10:OAc	
Spodoptera exigua	Noctuidae	Oocytes	SexiOR6	–	Liu et al., 2013a
			SexiOR11	–	
			SexiOR13	Z9,E12-14:OAc**, Z9-14:OAc*	
			SexiOR16	Z9-14:OH*	
Sesamia inferens	Noctuidae	Oocytes	SinfOR21	Z11-16:OH*	Zhang et al., 2014
			SinfOR29	Z11-16:OAc**	
			SinfOR27	Z9,E12-14:OAc	
Ctenopseustis obliquana	Tortricidae	HEK293	CoblOR7	Z8-14:OAc**	Steinwender et al., 2015
Ctenopseustis herana	Tortricidae	HEK293	CherOR7	Z8-14:OAc*, Z7-14:OAc**	Steinwender et al., 2015
Spodoptera litura	Noctuidae	Oocytes	SlituOR6	Z9,E12-14:OAc*	Zhang et al., 2015b
			SlituOR11	–	

(Continued)

TABLE 1 | Continued

Species	Family	Heterologous expression[a]	Genes[b]	Ligands	References
			SlituOR13	Z9-14:OAc*, Z9,E12-14:OAc	
			SlituOR16	Broadly tuned with the largest response to Z9-14:OH[c]	
Operophtera brumata	Geometridae	Oocytes	ObruOR1	1,Z3,Z6,Z9-19:H	Zhang et al., 2015a

[a] *Except for when specified differently in this column, the PRs were co-expressed with the respective Orco.*
[b] *HarmOR13, HarmOR11, HarmOR16 in Liu et al. (2013b) are equivalent genes to HarmOR1, HarmOR2 and HarmOR3 in Zhang et al. (2010), respectively; HassOR13 in Xu et al. (2015) is equivalent to HassOR1 in Zhang et al. (2010).*
[c] *Behavioral antagonist to corresponding species.*
**Minor pheromone components of corresponding species.*
***Major pheromone component of corresponding species.*
– No response to the tested compounds was observed.
Pheromone compounds are abbreviated in a standard way including (in order) geometry of the double bond, position of unsaturation, chain length followed by a colon and functionality. For example, E10,Z12-16:OH, (10E,12Z)-hexadecadienol; E10,Z12-16:Ald, (10E,12Z)-hexadecadienal; Z11-16:OAc, (11Z)-hexadecenyl acetate; and 1,Z3,Z6,Z9-19:H, (1,3Z,6Z,9Z)-nonadecatetraene.

effective mate recognition. On the other hand, following the asymmetric tracking hypothesis, males (the signal receivers) are under stronger selective pressures than females, and a subset of receptors with a broader response spectrum may serve as a preadaptation to be able to track variation in female-released pheromone signals (Phelan, 1997; Heckel, 2010; Wanner et al., 2010; Zhang and Löfstedt, 2013).

Some broadly tuned PRs are responsive to the behavioral antagonists. In this case a nonspecific neuron tuned to several antagonists might be sufficient to abort the flight toward the source (Takanashi et al., 2006), and the corresponding receptors may maintain a broad tuning profile instead of evolving specificity for a specific antagonist. Alternatively, as was recently found in *O. nubilalis*, a single OSN that respond to different behavioral antagonists may co-express multiple receptors. This might be another strategy for the moths to broaden the antagonism and increase the specificity of pheromone detection (Koutroumpa et al., 2014).

The phylogeny of the identified moth PRs reveals several apparent orthologous clusters (Cluster I–IV in **Figure 1B**) mainly expanded in the noctuids but also contain several genes from Bombycidae, Saturniidae, Geometridae, and Pyralidae. There are also some less defined clades expanded in the crambids, which contain PRs from Plutellidae and Tortricidae as well. Identification of PR genes from more Crambidae species may contribute to the recognition of orthologous clusters in these clades. PRs within the same orthologous cluster may respond to the same ligand, e.g., the HvOR13, HarmOR1, HassOR1, and AtraOR3 in Cluster IV are all specifically tuned to (11*Z*)-hexadecenal. However, the ligand profile of a candidate PR cannot be predicted simply by its orthology with known receptors. In clusters that have strong selective pressure indicated by a low dN/dS value, the PRs' ligand profiles tend to be conserved, whereas clusters with a high dN/dS value are relaxed from evolutionary constraint, thus have more divergent ligand profiles. In some species, paralogous PRs and their ligand profiles are more divergent compared to orthologous PRs (Zhang and Löfstedt, 2013). Because of the limited data of functionally characterized PRs, these patterns are put forward as hypotheses to be tested rather than conclusions.

In general, moth PR genes are under strong selective pressure to ensure the species-specific communication. It remains a conundrum how the moth PR functional diversity evolves under stabilizing selection. Gene duplication, which was suggested as an important mechanism for the diversification of olfactory receptors (Nei et al., 2008; Sánchez-Gracia et al., 2009), might also apply in PRs. Some closely related PR genes form a tightly linked cluster of duplicated genes as indicated by genetic mapping (Gould et al., 2010), and the PR paralogs arisen in the duplication events are under relaxed constraint, allowing the differentiation of their ligand preference (Zhang and Löfstedt, 2013). Another possible mechanism might be that the common ancestor of current orthologous PRs was broadly tuned, and later selected to respond specifically to certain pheromone compounds in different species.

Future Research on Moth PRs

With the facility of transcriptome sequencing, it is now straightforward to obtain the sequences of candidate PRs. Since most of the PRs identified to date are from noctuid species that normally use fatty acyl alcohol, aldehyde and acetate pheromone compounds, it would be interesting to broaden the search to explore the PRs tuned to other type of pheromones, such as the Type II long chain polyenes and epoxides, or the short chain ketones and secondary alcohols that are used as pheromones in more basal lepidopteran families (Löfstedt and Kozlov, 1997).

The mechanisms underlying ligand selectivity within a receptor still remain largely unclear. Determination of the key amino acids in the ligand-binding region may help to clarify what determines specificity. Comparison of orthologous PRs with different pheromone specificities, or with the same ligand specificity in evolutionary distant species, as well as mutagenesis of the sites under positive selection (Leary et al., 2012) will help to identify the amino acids of importance to the receptor-ligand interaction. Solution of the crystal structures of pheromone receptors, a major challenge due to the

technical difficulties of working with membrane proteins, may ultimately provide the information necessary to test hypotheses concerning the relationship between receptor sequence and specificity, as well as the interaction between PR and the co-receptor.

The transduction of sex pheromone signals has been intensively investigated since the early days of pheromone research and remains a hotspot of current research effort on PRs. Research has focused on the formation of the heteromeric ligand-gated non-selective ion channels through the combination of Orco and PRs (e.g., Nakagawa et al., 2005; Wicher et al., 2008), the binding and transport of the target sex pheromone components to the OSN's dendrites (Vogt, 2005; Sato et al., 2008), as well as the close association of PRs and SNMPs (Benton et al., 2007; Jin et al., 2008; Li et al., 2014; Pregitzer et al., 2014). Progress on these fundamental questions will greatly enrich our understanding of the working mechanism of moth PRs.

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References

Ando, T., Inomate, S. I., and Yamamoto, M. (2004). "Lepidopteran sex pheromones," in *The Chemistry of Pheromones and Other Semiochemicals*: *Vol. 239, Topics in Current Chemistry*, ed S. Schulz (New York, NY: Springer), 51–96. doi: 10.1007/b95449

Baker, T. C. (2008). Balanced olfactory antagonism as a concept for understanding evolutionary shifts in moth sex pheromone blends. *J. Chem. Ecol.* 34, 971–981. doi: 10.1007/s10886-008-9468-5

Baker, T. C. (2009). Nearest neural neighbors: moth sex pheromone receptors HR11 and HR13. *Chem. Senses* 34, 465–468. doi: 10.1093/chemse/bjp025

Bengtsson, J. M., Trona, F., Montagné, N., Anfora, G., Ignell, R., Witzgall, P., et al. (2012). Putative chemosensory receptors of the codling moth, *Cydia pomonella*, identified by antennal transcriptome analysis. *PLoS ONE* 7:e31620. doi: 10.1371/journal.pone.0031620

Benton, R., Vannice, K. S., and Vosshall, L. B. (2007). An essential role for a CD36-related receptor in pheromone detection in *Drosophila*. *Nature* 450, 289–293. doi: 10.1038/nature06328

Cardé, R. T., and Haynes, K. F. (2004). "Structure of the pheromone communication channel in moths," in *Advances in Insect Chemical Ecology*, ed R. T. Cardé and J. G. Millar (Cambridge: Cambridge University Press), 283–332. doi: 10.1017/cbo9780511542664.009

Clyne, P. J., Warr, C. G., Freeman, M. R., Lessing, D., Kim, J., and Carlson, J. R. (1999). A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron* 22, 327–338. doi: 10.1016/S0896-6273(00)81093-4

Corcoran, J. A., Jordan, M. D., Carraher, C., and Newcomb, R. D. (2014). A novel method to study insect olfactory receptor function using HEK293 cells. *Insect Biochem. Mol. Biol.* 54, 22–32. doi: 10.1016/j.ibmb.2014.08.005

Forstner, M., Breer, H., and Krieger, J. (2009). A receptor and binding protein interplay in the detection of a distinct pheromone component in the silkmoth *Antheraea polyphemus*. *Int. J. Biol. Sci.* 5, 745–757. doi: 10.7150/ijbs.5.745

Gould, F., Estock, M., Hillier, N. K., Powell, B., Groot, A. T., Ward, C. M., et al. (2010). Sexual isolation of male moths explained by a single pheromone response QTL containing four receptor genes. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8660–8665. doi: 10.1073/pnas.0910945107

Große-Wilde, E., Gohl, T., Bouché, E., Breer, H., and Krieger, J. (2007). Candidate pheromone receptors provide the basis for the response of distinct antennal neurons to pheromonal compounds. *Eur. J. Neurosci.* 25, 2364–2373. doi: 10.1111/j.1460-9568.2007.05512.x

Große-Wilde, E., Kuebler, L. S., Bucks, S., Vogel, H., Wicher, D., and Hansson, B. S. (2011). Antennal transcriptome of *Manduca sexta*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 7449–7454. doi: 10.1073/pnas.1017963108

Große-Wilde, E., Svatoš, A., and Krieger, J. (2006). A pheromone-binding protein mediates the bombykol-induced activation of a pheromone receptor *in vitro*. *Chem. Senses* 31, 547–555. doi: 10.1093/chemse/bjj059

Ha, T. S., and Smith, D. P. (2006). A pheromone receptor mediates 11-*cis*-vaccenyl acetate-induced responses in *Drosophila*. *J. Neurosci.* 26, 8727–8733. doi: 10.1523/JNEUROSCI.0876-06.2006

Hamada, S., Tabuchi, M., Toyota, T., Sakurai, T., Hosoi, T., Nomoto, T., et al. (2014). Giant vesicles functionally expressing membrane receptors for an insect pheromone. *Chem. Commun.* 50, 2958–2961. doi: 10.1039/c3cc48216b

Heckel, D. G. (2010). Smells like a new species: gene duplication at the periphery. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9481–9482. doi: 10.1073/pnas.1004511107

Jin, X., Ha, T. S., and Smith, D. P. (2008). SNMP is a signaling component required for pheromone sensitivity in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10996–11001. doi: 10.1073/pnas.0803309105

Koutroumpa, F. A., Kárpáti, Z., Monsempes, C., Hill, S. R., Hansson, B. S., Jacquin-Joly, E., et al. (2014). Shifts in sensory neuron identity parallel differences in pheromone preference in the European corn borer. *Front. Ecol. Evol.* 2:65. doi: 10.3389/fevo.2014.00065

Krieger, J., Gondesen, I., Forstner, M., Gohl, T., Dewer, Y., and Breer, H. (2009). HR11 and HR13 receptor-expressing neurons are housed together in pheromone-responsive sensilla trichodea of male *Heliothis virescens*. *Chem. Senses* 34, 469–477. doi: 10.1093/chemse/bjp012

Krieger, J., Große-Wilde, E., Gohl, T., and Breer, H. (2005). Candidate pheromone receptors of the silkmoth *Bombyx mori*. *Eur. J. Neurosci.* 21, 2167–2176. doi: 10.1111/j.1460-9568.2005.04058.x

Krieger, J., Große-Wilde, E., Gohl, T., Dewer, Y. M., Raming, K., and Breer, H. (2004). Genes encoding candidate pheromone receptors in a moth (*Heliothis virescens*). *Proc. Natl. Acad. Sci. U.S.A.* 101, 11845–11850. doi: 10.1073/pnas.0403052101

Kurtovic, A., Widmer, A., and Dickson, B. J. (2007). A single class of olfactory neurons mediates behavioural responses to a *Drosophila* sex pheromone. *Nature* 446, 542–546. doi: 10.1038/nature05672

Le, S. Q., and Gascuel, O. (2008). An improved general amino acid replacement matrix. *Mol. Biol. Evol.* 25, 1307–1320. doi: 10.1093/molbev/msn067

Leary, G. P., Allen, J. E., Bunger, P. L., Luginbill, J. B., Linn, C. E. Jr., Macallister, I. E., et al. (2012). Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14081–14086. doi: 10.1073/pnas.1204661109

Li, Z., Ni, J. D., Huang, J., and Montell, C. (2014). Requirement for *Drosophila* SNMP1 for rapid activation and termination of pheromone-induced activity. *PLoS Genet.* 10:e1004600. doi: 10.1371/journal.pgen.1004600

Linn, C. E, Jr., Domingue, M. J., Musto, C. J., Baker, T. C., and Roelofs, W. L. (2007). Support for (*Z*)-11-hexadecanal as a pheromone antagonist in *Ostrinia nubilalis*: flight tunnel and single sensillum studies with a New York population. *J. Chem. Ecol.* 33, 909–921. doi: 10.1007/s10886-007-9268-3

Linn, C. E., and Roelofs, W. L. (1995). "Pheromone communication in the moths and its role in the speciation process," in *Speciation and the Recognition Concept: Theory and Application*, ed D. Lambert and H. Spencer (Baltimore, MD: Johns Hopkins University Press), 263–300.

Liu, C., Liu, Y., Walker, W. B., Dong, S., and Wang, G. (2013a). Identification and functional characterization of sex pheromone receptors in beet armyworm *Spodoptera exigua* (Hübner). *Insect Biochem. Mol. Biol.* 43, 747–754. doi: 10.1016/j.ibmb.2013.05.009

Liu, Y., Liu, C., Lin, K., and Wang, G. (2013b). Functional specificity of sex pheromone receptors in the cotton bollworm *Helicoverpa armigera*. *PLoS ONE* 8:e62094. doi: 10.1371/journal.pone.0062094

Löfstedt, C., and Kozlov, M. (1997). "A phylogenetic analysis of pheromone communication in primitive moths," in *Insect Pheromone Research: New Directions*, eds R. T. Cardé and A. K. Mink (New York, NY: Chapman & Hall), 473–489. doi: 10.1007/978-1-4615-6371-6_41

Millar, J. G. (2000). Polyene hydrocarbons and epoxides: a second major class of lepidopteran sex attractant pheromones. *Annu. Rev. Entomol.* 45, 575–604. doi: 10.1146/annurev.ento.45.1.575

Mitsuno, H., Sakurai, T., Murai, M., Yasuda, T., Kugimiya, S., Ozawa, R., et al. (2008). Identification of receptors of main sex-pheromone components of three Lepidopteran species. *Eur. J. Neurosci.* 28, 893–902. doi: 10.1111/j.1460-9568.2008.06429.x

Miura, N., Nakagawa, T., Tatsuki, S., Touhara, K., and Ishikawa, Y. (2009). A male-specific odorant receptor conserved through the evolution of sex pheromones in *Ostrinia* moth species. *Int. J. Biol. Sci.* 5, 319–330. doi: 10.7150/ijbs.5.319

Miura, N., Nakagawa, T., Touhara, K., and Ishikawa, Y. (2010). Broadly and narrowly tuned odorant receptors are involved in female sex pheromone reception in *Ostrinia* moths. *Insect Biochem. Mol. Biol.* 40, 64–73. doi: 10.1016/j.ibmb.2009.12.011

Montagné, N., Chertemps, T., Brigaud, I., François, A., François, M. C., de Fouchier, A., et al. (2012). Functional characterization of a sex pheromone receptor in the pest moth *Spodoptera littoralis* by heterologous expression in *Drosophila*. *Eur. J. Neurosci.* 36, 2588–2596. doi: 10.1111/j.1460-9568.2012.08183.x

Montagné, N., de Fouchier, A., Newcomb, R. D., and Jacquin-Joly, E. (2015). Advances in the identification and characterization of olfactory receptors in insects. *Prog. Mol. Biol. Transl. Sci.* 130, 55–80. doi: 10.1016/bs.pmbts.2014.11.003

Nakagawa, T., Pellegrino, M., Sato, K., Vosshall, L. B., and Touhara, K. (2012). Amino acid residues contributing to function of the heteromeric insect olfactory receptor complex. *PLoS ONE* 7:e32372. doi: 10.1371/journal.pone.0032372

Nakagawa, T., Sakurai, T., Nishioka, T., and Touhara, K. (2005). Insect sex pheromone signals mediated by specific combinations of olfactory receptors. *Science* 307, 1638–1642. doi: 10.1126/science.1106267

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. doi: 10.1038/nrg2480

Phelan, P. L. (1997). "Genetics and phylogenetics in the evolution of sex pheromones," in *Insect Pheromone Research: New Directions*, eds R. T. Cardé and A. K. Minks (New York, NY: Chapman & Hall), 563–579.

Poivet, E., Gallot, A., Montagne, N., Glaser, N., Legeai, F., and Jacquin-Joly, E. (2013). A comparison of the olfactory gene repertoires of adults and larvae in the noctuid moth *Spodoptera littoralis*. *PLoS ONE* 8:e60263. doi: 10.1371/journal.pone.0060263

Pregitzer, P., Greschista, M., Breer, H., and Krieger, J. (2014). The sensory neurone membrane protein SNMP1 contributes to the sensitivity of a pheromone detection system. *Insect Mol. Biol.* 23, 733–742. doi: 10.1111/imb.12119

Roelofs, W. L., Hill, A. S., Linn, C. E., Meinwald, J., Jain, S. C., Herbert, H. J., et al. (1982). Sex pheromone of the winter moth, a geometrid with unusually low temperature precopulatory responses. *Science* 217, 657–659. doi: 10.1126/science.217.4560.657

Sakurai, T., Nakagawa, T., Mitsuno, H., Mori, H., Endo, Y., Tanoue, S., et al. (2004). Identification and functional characterization of a sex pheromone receptor in the silkmoth *Bombyx mori*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16653–16658. doi: 10.1073/pnas.0407596101

Sánchez-Gracia, A., Vieira, F. G., and Rozas, J. (2009). Molecular evolution of the major chemosensory gene families in insects. *Heredity* 103, 208–216. doi: 10.1038/hdy.2009.55

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. doi: 10.1038/nature06850

Schneider, D. (1974). The sex-attractant receptor of moths. *Sci. Am.* 231, 28–35. doi: 10.1038/scientificamerican0774-28

Steinwender, B., Thrimawithana, A. H., Crowhurst, R. N., and Newcomb, R. D. (2015). Pheromone receptor evolution in the cryptic leafroller species, *Ctenopseustis obliquana* and *C. herana*. *J. Mol. Evol.* 80, 42–56. doi: 10.1007/s00239-014-9650-z

Sun, M., Liu, Y., Walker, W. B., Liu, C., Lin, K., Gu, S., et al. (2013). Identification and characterization of pheromone receptors and interplay between receptors and pheromone binding proteins in the diamondback moth, *Plutella xyllostella*. *PLoS ONE* 8:e62098. doi: 10.1371/journal.pone.0062098

Syed, Z., Ishida, Y., Taylor, K., Kimbrell, D. A., and Leal, W. S. (2006). Pheromone reception in fruit flies expressing a moth's odorant receptor. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16538–16543. doi: 10.1073/pnas.0607874103

Syed, Z., Kopp, A., Kimbrell, D. A., and Leal, W. S. (2010). Bombykol receptors in the silkworm moth and the fruit fly. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9436–9439. doi: 10.1073/pnas.1003881107

Takanashi, T., Ishikawa, Y., Anderson, P., Huang, Y., Löfstedt, C., Tatsuki, S., et al. (2006). Unusual response characteristics of pheromone-specific olfactory receptor neurons in the Asian corn borer moth, *Ostrinia furnacalis*. *J. Exp. Biol.* 209, 4946–4956. doi: 10.1242/jeb.02587

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197

Vogt, R. G. (2005). "Molecular basis of pheromone detection in insects," in *Comprehensive Insect Physiology, Biochemistry, Pharmacology and Molecular Biology*, Vol. 3, eds L. I. Gilbert, K. Iatrou, and S. S. Gill (Oxford: Elsevier Pergamon), 753–803.

Vosshall, L. B., Amrein, H., Morozov, P. S., Rzhetsky, A., and Axel, R. (1999). A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell* 96, 725–736. doi: 10.1016/S0092-8674(00)80582-6

Wang, G., Vásquez, G. M., Schal, C., Zwiebel, L. J., and Gould, F. (2011). Functional characterization of pheromone receptors in the tobacco budworm *Heliothis virescens*. *Insect Mol. Biol.* 20, 125–133. doi: 10.1111/j.1365-2583.2010.01045.x

Wanner, K. W., Nichols, A. S., Allen, J. E., Bunger, P. L., Garczynski, S. F., Linn, C. E., et al. (2010). Sex pheromone receptor specificity in the European corn borer moth, *Ostrinia nubilalis*. *PLoS ONE* 5:e8685. doi: 10.1371/journal.pone.0008685

Wicher, D., Schäfer, R., Bauernfeind, R., Stensmyr, M. C., Heller, R., Heinemann, S. H., et al. (2008). *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature* 452, 1007–1011. doi: 10.1038/nature06861

Xu, P., Garczynski, S. F., Atungulu, E., Syed, Z., Choo, Y. M., Vidal, D. M., et al. (2012). Moth sex pheromone receptors and deceitful parapheromones. *PLoS ONE* 7:e41653. doi: 10.1371/journal.pone.0041653

Xu, W., Papanicolaou, A., Liu, N. Y., Dong, S. L., and Anderson, A. (2015). Chemosensory receptor genes in the oriental tobacco budworm *Helicoverpa assulta*. *Insect Mol. Biol.* 24, 253–263. doi: 10.1111/imb.12153

Zhang, D. D. (2010). *Cloning And Functional Identification Of Pheromone Receptor Genes in Two Sibling Moth Species, Helicoverpa Armigera And Helicoverpa Assulta*. Ph. D. thesis, Institute of Zoology, Chinese Academy of Sciences, Beijing.

Zhang, D. D., and Löfstedt, C. (2013). Functional evolution of a multigene family: orthologous and paralogous pheromone receptor genes in the turnip moth, *Agrotis segetum*. *PLoS ONE* 8:e77345. doi: 10.1371/journal.pone.0077345

Zhang, D. D., Wang, H. L., Schultze, A., Froß, H., Francke, W., Krieger, J., et al. (2015a). "Functional characterization of a receptor for a Type II sex pheromone in the winter moth, Operophtera brumata (Lepidoptera: Geometridae)," *The 31st Annual Meeting of the International Society of Chemical Ecology* (Stockholm), 81.

Zhang, D. D., Zhu, K. Y., and Wang, C. Z. (2010). Sequencing and characterization of six cDNAs putatively encoding three pairs of pheromone receptors in two

sibling species, *Helicoverpa armigera* and *Helicoverpa assulta*. *J. Insect. Physiol.* 56, 586–593. doi: 10.1016/j.jinsphys.2009.12.002

Zhang, J., Yan, S., Liu, Y., Jacquin-Joly, E., Dong, S., and Wang, G. (2015b). Identification and functional characterization of sex pheromone receptors in the common cutworm (*Spodoptera litura*). *Chem. Senses* 40, 7–16. doi: 10.1093/chemse/bju052

Zhang, Y. N., Zhang, J., Yan, S. W., Chang, H. T., Liu, Y., Wang, G. R., et al. (2014). Functional characterization of sex pheromone receptors in the purple stem borer, *Sesamia inferens* (Walker). *Insect Mol. Biol.* 23, 611–620. doi: 10.1111/imb.12109

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Evolution of two receptors detecting the same pheromone compound in crop pest moths of the genus *Spodoptera*

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In moths, mate finding strongly rely on the detection of sex pheromones by pheromone receptors (PRs). Any modification in the functional properties of these receptors can have a drastic impact on reproduction. In the course of characterizing candidate PRs in the noctuid moth *Spodoptera littoralis*, we expressed them in *Drosophila* olfactory sensory neurons and stimulated them with a large panel of moth pheromone compounds. We found that two PRs detect (*Z*,*E*)-9,12-14:OAc, a minor component of the female pheromone blend. Whereas SlitOR6 is highly specific to this component, SlitOR13 is less sensitive and not strictly specific as it also detects (*Z*)9-14:OAc, another minor component of the sex pheromone. Interestingly, SlitOR13 expression is restricted to the distal part of male antennae, where we could identify a novel functional class of pheromone-sensitive neurons whose response spectrum matches that of SlitOR13. Based on a phylogenetic analysis of Lepidoptera PRs, we found that the ability to bind (*Z*,*E*)-9,12-14:OAc appeared independently within three paralogous lineages, and an analysis of selective pressures revealed sites under positive selection that could have played a role in the emergence of functional properties of OR6 and OR13 in *Spodoptera* species.

Keywords: insect, olfaction, olfactory receptor, sex pheromone, *Spodoptera littoralis*, heterologous expression, positive selection

Introduction

In animals, various biological mechanisms prevent species from interbreeding with each other. One of the well-studied mechanisms of premating isolation is the sex pheromone communication system of moths (Cardé and Haynes, 2004). In those nocturnal insects, reproductive success largely depends on the long-distance detection of bouquets of air-borne chemicals usually emitted by the females. Most sex pheromones consist of a complex blend of a major component mixed with a few minor components, whose nature and precise relative ratios ensure a species-specific recognition (de Bruyne and Baker, 2008). In the male, antennae bear thousands of sensilla housing olfactory sensory neurons (OSNs) that detect the different components of the pheromone blend with various

sensitivities and specificities (Kaissling, 1996). At the cellular level, this detection is mediated by transmembrane receptors belonging to the olfactory receptor (OR) family, and called pheromone receptors (PRs). Moth PR-encoding genes form a monophyletic group in the OR phylogeny (de Fouchier et al., 2014), suggesting that they all evolved from a single common ancestor. Numerous gene duplication events led to the variable number of putative PRs (usually from 4 to 9) found in the different species investigated (Krieger et al., 2004; Nakagawa et al., 2005; Wanner et al., 2010; Grosse-Wilde et al., 2011; Bengtsson et al., 2012; Zhang and Lofstedt, 2013; Steinwender et al., 2015).

The functional evolution of PRs is a key issue, because even slight modifications of their receptive range are expected to give rise to new reproductive barriers between sympatric populations, then to the emergence of new species. Since the discovery of the receptor for bombykol in the silk moth *Bombyx mori* (Sakurai et al., 2004), ligands of PRs have been identified in more than a dozen of species (for a review, see de Fouchier et al., 2014). These functional data highlight a rapid functional divergence among moth PRs resulting, at least in part, from increased evolutionary rates following gene duplications (Zhang and Lofstedt, 2013; Engsontia et al., 2014). At an extreme, some receptors belonging to the PR sub-family do not bind sex pheromone components but rather plant volatiles (Jordan et al., 2009; Bengtsson et al., 2014). Whereas functional properties are generally more conserved between orthologous genes from closely related species, only a few amino acid changes can modify these properties, sometimes drastically (Leary et al., 2012; Jiang et al., 2014; Steinwender et al., 2015).

Moths from the genus *Spodoptera* (Lepidoptera, Noctuidae) form a group of economically important crop pests. Several species are sympatric and synthesize partially overlapping pheromone blends, which make them an ideal model to study the links between the evolution of PRs and premating isolation. One of the most studied *Spodoptera* species is the cotton leafworm *S. littoralis*. It is a serious pest of more than 80 agricultural productions such as cotton, maize, rice, sorghum, alfalfa, soybean and vegetables, all over Africa, the Mediterranean Basin and the Middle East (Salama et al., 1971). Its sex pheromone blend, variable according to the strains and areas, is composed of up to eleven 14-carbon acetates, among which (*Z*,*E*)-9,11-14:OAc is always the major component (Muñoz et al., 2008; Saveer et al., 2014). Electrophysiology experiments on male antennae identified one OSN population specifically tuned to this component (Ljungberg et al., 1993; Quero et al., 1996) and housed in one class of long trichoid sensilla, further referred as LT1 sensilla. Another functional class of sensilla, here referred as LT2, house two OSNs, one of which tuned to the minor component (*Z*,*E*)-9,12-14:OAc (Ljungberg et al., 1993). As far as we know, no OSN detecting other *S. littoralis* pheromone components could be identified.

We previously identified four candidate PRs (named SlitOR6, 11, 13, and 16) in a *S. littoralis* male transcriptome, based on their clustering with other moth PRs in a phylogenetic analysis (Legeai et al., 2011). Among them, SlitOR6 has been characterized as a receptor for the minor component (*Z*,*E*)-9,12-14:OAc (Montagné et al., 2012). Through homology-cloning, the orthologs of these four PRs have been identified in *S. litura* (found throughout Asia and Oceania) and *S. exigua* (distributed worldwide) and functional data are available for some of them (Liu et al., 2013a; Zhang et al., 2015).

Here, we carried out a functional analysis of the four *S. littoralis* PR candidates, using a large panel of pheromone compounds, to investigate in depth the response specificity. We used *in vivo* heterologous expression in *Drosophila* OSNs housed in trichoid sensilla, an expression system that has been demonstrated to be suitable for studying moth PRs (Kurtovic et al., 2007; Syed et al., 2010; Montagné et al., 2012; Bengtsson et al., 2014). We identified two receptors to minor components with overlapping response spectra, both responding to (*Z*,*E*)-9,12-14:OAc, and localized the corresponding OSNs on male moth antenna. Evolutionary analyses revealed that the ability to bind (*Z*,*E*)-9,12-14:OAc emerged independently within three distinct paralogous lineages, two of which containing *Spodoptera spp*. sequences, and that positive selection acted on a few amino acid sites, which could have played a role in the evolution of PR response spectra in *Spodoptera* species.

Materials and Methods

Insect Rearing and Chemicals

Flies were reared on standard cornmeal-yeast-agar medium and kept in a climate- and light-controlled environment (25°C, 12 h light: 12 h dark cycle). *S. littoralis* were reared in the laboratory on a semi-artificial diet (Poitout and Buès, 1974) at 22°C, 60% relative humidity and under a 16 h light: 8 h dark cycle. Males and females were sexed as pupae and further reared separately. The pheromone compounds used in this study (see Supplementary Table S1) were either synthesized in the lab or purchased from Sigma-Aldrich (St Louis, MO, USA) and Pherobank (Wijk bij Duurstede, The Netherlands). Hexane was purchased from Carlo Erba Reagents (Val de Reuil, France).

Heterologous Expression of Slitors in *Drosophila*

The generation of transgenic flies expressing SlitOR6 has been described previously (Montagné et al., 2012). The same strategy was used for expression of SlitOR11, 13 and 16. Briefly, full-length open reading frames were first cloned into pCR®II-TOPO® (Invitrogen, Carlsbad, CA, USA) and subcloned into the pUAST vector. Plasmid constructs were purified from liquid cultures of One Shot® TOP10 *E. coli* using the EndoFree Plasmid Maxi kit (Qiagen, Venlo, Netherlands). Transformant *UAS-SlitOr* balanced fly lines were generated by BestGene Inc. (Chino Hills, California, USA), by injecting the pUAST-SlitOR plasmids into w^{1118} fly embryos. Only fly lines harboring a transgene insertion into the 2nd chromosome were used for further crossings.

UAS-SlitOr balanced lines were crossed to a line harboring a knock-in of the *Gal4* ORF into the *Or67d* gene (Kurtovic et al., 2007), to obtain double homozygous flies (genotype *w*; *UAS-SlitOr*,w^+; $Or67d^{Gal4}$) expressing a given SlitOR in at1 OSNs instead of the endogenous *Drosophila* receptor OR67d. The presence of the *UAS-SlitOr* transgenes was verified by PCR on genomic DNA extracted from two flies, and the correct expression of the SlitORs was verified by RT-PCR on total RNA extracted from ≥100 pairs of antennae.

Single-sensillum Recordings on *Drosophila* Antennae

Single-sensillum recordings were performed on at1 sensilla of transformed flies expressing a given SlitOR. For each experiment, a 2- to 6-day-old male fly was restrained in a pipette tip with only the head protruding. The tip was fixed on a microscope glass slide and one antenna was gently maintained using a glass capillary. The preparation was placed under a constant 1.5 $L.min^{-1}$ flux of charcoal-filtered and humidified air delivered through a glass tube of a 7 mm diameter, and observed with a light microscope (BX51WI, Olympus, Tokyo, Japan) equipped with a 100× magnification objective.

The SlitOR response spectra were established using a panel of 26 pheromone compounds (Supplementary Table S1). Stimulus cartridges were built by placing a 1 cm^2 filter paper in the large opening end of a Pasteur pipette and dropping 10 µl of the pheromone solution onto the paper (1 µg/µl in hexane), or 10 µL of hexane as control. The cartridges were used at most one time on each fly and five times in total. Odorant stimulations were performed by inserting the tip of the pipette into a hole in the glass tube and generating a 500 ms air pulse (0.6 $L.min^{-1}$), which reached the permanent air flux while going through the stimulation cartridge. Action potentials were recorded from at1 sensilla using electrolytically sharpened tungsten electrodes (TW5-6, Science Products, Hofheim, Germany). The reference electrode was inserted into the eye and the recording electrode was inserted at the base of the sensillum using a motor-controlled PatchStar micromanipulator (Scientifica, Uckfield, United Kingdom). The electrical signal was amplified using an EX-1 amplifier (Dagan Corporation, Minneapolis, MN, USA), high-pass (1 Hz) and low-pass (3 kHz) filtered and digitized (10 kHz) through a Digidata 1440A acquisition board (Molecular Devices, Sunnyvale, CA, USA) then recorded and analyzed using the pCLAMP™ 10 software (Molecular Devices). The responses of at1 OSNs were calculated by subtracting the spontaneous firing rate (in $spikes.s^{-1}$) from the firing rate during the odorant stimulation. The time windows used to measure these two firing rates lasted for 500 ms and were respectively placed 500 ms before and 100 ms after the onset of stimulation (to take into account the time for the odorants to reach the antenna). The entire odorant panel was tested at least 5 times on each SlitOR. Odorants were considered as active if the response they elicited was statistically different from the response elicited by the solvent alone ($P < 0.001$; Mann–Whitney pairwise test). For all active odorants, dose-response experiments were conducted with quantities ranging from 10 µg down to the dose necessary to reach the response threshold. Each dilution of each pheromone was tested five times.

Single-sensillum Recordings on *Spodoptera* Antennae

Recordings of *S. littoralis* long trichoid sensilla were performed on 1- to 3-day-old male moths. Animals were restrained in a Styrofoam block and the antenna was visualized under a MZ16 stereomicroscope (Leica, Wetzlar, Germany). A humidified and charcoal-filtered airflow (1.2 $L.min^{-1}$) was continuously directed to the preparation through a glass tube (7 mm of diameter). The stimulus panel consisted in the same 26 pheromone compounds as for recordings on *Drosophila* antennae (Supplementary Table S1). Stimulus cartridges were prepared as described above and were loaded with 1 µg of pheromone. During the stimulation, the tip of the Pasteur pipette was introduced through a lateral hole within the tube carrying the permanent humidified air flow onto the antennae at 15 cm from its outlet. The air pulse (0.2 $L.min^{-1}$) lasted for 200 ms. Tungsten electrodes were prepared as described above and the recording electrode was inserted at the base of the sensillum of interest using a PatchStar micromanipulator (Scientifica). The biological signal was amplified (×2000), high-pass (1 Hz) and low-pass (3 kHz) filtered using a CyberAmp 320 (Molecular Devices) and sampled at 10 kHz via a Digidata 1440A acquisition board (Molecular Devices). Recordings and analyses were performed with pCLAMP™ 10 (Molecular Devices). The responses were calculated as described above, using time windows of 200 ms. The stimulus panel was tested 3 times on each of the two functional classes of long trichoid sensilla investigated. For the cross adaptation experiment, sensilla were first stimulated during 5 s with either (*Z*)9-12:OAc, (*Z*)9-14:OAc or (*Z*,*E*)-9,12-14:OAc, then stimulated during 200 ms with the three compounds presented successively in a random order.

Quantitative Real-time PCR

Antennae from 2-day-old males were collected and cut into three pieces: a proximal part, a middle part and a distal part. Proximal parts from 60 individuals were pooled, as well as distal parts. These collections were repeated three times (biological replicates). Total RNA was extracted from each sample using RNeasy MicroKit (Qiagen), which included a DNase treatment. cDNA was synthesized using the Advantage® RT-for-PCR Kit (Clontech, Mountain View, CA, USA). Gene-specific primers for SlitOR6, SlitOR13 and the reference gene SlitORco have been previously described (Legeai et al., 2011). We used ORco, expected to be expressed equally in all OSNs (Larsson et al., 2004), as the reference gene because the number of sensilla (and thus the number of OSNs) clearly differs between the proximal (higher number of sensilla) and the distal parts (lower number of sensilla) of the antennae. Thus, normalization with a housekeeping gene would have biased relative expression calculation. qPCR mix was prepared in a total volume of 12 µL with 6 µL of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 3 µL of diluted cDNA (or water for the negative control or RNA for controlling the absence of genomic DNA) and 200 nM of each primer. qPCR assays were performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The PCR program began with a cycle at 95°C for 3 min, followed by 40 cycles of 10 s at 95°C, 30 s at 60°C. To assess the specificity of the PCR reactions, a dissociation curve of the amplified products was performed by gradual heating from 65°C to 95°C at $0.5°C.s^{-1}$. Standard curves were generated by a five-fold dilution series of a cDNA pool evaluating primer efficiency ($E = 10^{(-1/slope)}$). For each case, the presence of only one amplified product was verified. All reactions were performed in duplicate for the three biological replicates. Expression levels between proximal and

distal parts of the antennae were calculated relatively to the expression of the reference gene using the formula ratio = $[(E_{target})^{\Delta Cp\,target\,(control-sample)}]/[(E_{ref})^{\Delta Cp\,ref\,(control-sample)}]$ (Pfaffl, 2001).

Phylogenetic Analysis

An amino acid sequence dataset was created, including every full-length candidate PR sequence identified in Lepidoptera (see Supplementary Table S2). *Bombyx mori* OR6 and *Ctenopseustis obliquana* OR22 were also included to serve as an external group. The 149 amino acid sequences were aligned using the online version of MAFFT v.7 (Katoh and Standley, 2013), with the G-INS-i algorithm (Katoh et al., 2005) and default parameters. Phylogenetic reconstruction was performed using the maximum likelihood method. The JTT+I+G+F substitution model (Jones et al., 1992), was determined as the best-fit model of protein evolution by ProtTest 2.4 (Abascal et al., 2005). Tree reconstruction was performed using PhyML 3.0 (Guindon et al., 2010), with both SPR (Subtree Pruning and Regrafting) and NNI (Nearest Neighbor Interchange) methods for topology improvement. Rate heterogeneity was set at four categories, and values calculated by ProtTest were used for the gamma distribution parameter and the proportion of invariable sites. Node support was estimated using a hierarchical likelihood-ratio test (Anisimova and Gascuel, 2006). The figure was created using the iTOL web server (Letunic and Bork, 2011).

Analysis of Selective Pressures

To study positive selection along the PR alignment, we first manually removed the sites which were poorly aligned or conserved. Next, we replaced the amino acids by their codons, and created a DNA alignment file that corresponded to the trimmed protein alignment. From that DNA alignment, we constructed a phylogenetic tree using PhyML 3.0 with the GTR substitution model, 4 substitution rate categories and an estimated gamma shape parameter. Both SPR and NNI-types tree modifications were allowed. Branch support values were generated using the Shimodaira-Hasegawa test (Shimodaira, 2002) as implemented in PhyML. We then used this tree and both protein and DNA alignments of OR sequences to perform branch-site tests of positive selection, which have proven to be more sensitive than branch-based tests, in regions where alignments are reliable (Yang and dos Reis, 2011). To do so we used the software fastcodeml (Valle et al., 2014), a recent program designed to reduce the calculation time of branch-site models developed in the codeml suite of programs (formerly defined in codeml using the parameters model = 2 and NSsites = 2). We split the full tree up into 6 independent clades having large support values (>0.95) and we performed the selection tests on each one of them. We made full use of fastcodeml parallelized algorithms by specifying computations with 8 threads on a computer cluster (parameter –nt 8). For each of the clades we reported the branches that have at least one site under positive selection and noted the associated probability that a diversifying selection occurred at this site along the branch under consideration.

Results

Response Spectra of Heterologously Expressed SlitORs

In order to systematically analyze the response spectra of SlitOR6, 11, 13 and 16 to a large panel of moth pheromone compounds, we expressed these receptors in *Drosophila* OSNs housed in at1 trichoid sensilla, in place of the endogenous PR DmelOR67d. RT-PCR experiments confirmed the correct expression of the SlitORs, except SlitOR16 (data not shown) that has not been studied further. Using the single-sensillum recording technique, we monitored the response of OSNs expressing SlitOR6, 11 and 13 to high doses of 26 pheromone compounds (Supplementary Table S1). This panel included all the compounds identified in the pheromone blend of *S. littoralis* and/or active on *S. littoralis* antennae (Ljungberg et al., 1993; Muñoz et al., 2008; Saveer et al., 2014), as well as closely related chemicals and ligands of previously characterized noctuid PRs (Wang et al., 2011). While SlitOR11-expressing OSNs did not display any response to the panel (data not shown), SlitOR6 and SlitOR13-expressing OSNs gave significant responses to two and three compounds, respectively (**Figure 1A**). As observed previously (Montagné et al., 2012), SlitOR6 responded strongly to (*Z,E*)-9,12-14:OAc (133 spikes.s^{-1}, $P < 0.001$, Mann–Whitney). Here, we also observed a smaller response to (*Z*)9-12:OAc (35 spikes.s^{-1}, $P < 0.01$). SlitOR13 displayed similar responses to (*Z,E*)-9,12-14:OAc and (*Z*)9-14:OAc (76 and 77 spikes.s^{-1}, $P < 0.001$), as well as a smaller response to (*Z*)9-12:OAc (52 spikes.s^{-1}, $P < 0.001$).

To further analyze both the sensitivity and the selectivity of SlitOR6 and 13, we performed dose-response experiments with the ligands identified among the panel (**Figure 2**). The response of SlitOR6 to (*Z*)9-12:OAc was abolished at a dose of 1 μg loaded in the stimulus cartridge, while it still responded to (*Z,E*)-9,12-14:OAc at 0.1 μg ($P < 0.05$). SlitOR13 responded to (*Z*)9-14:OAc and (*Z,E*)-9,12-14:OAc down to 1 μg, with similar intensities. We thus identified two PRs detecting the same minor component of the *S. littoralis* pheromonal bouquet, albeit with different sensitivity and specificity.

Identification of Two Different *S. littoralis* OSN Types Tuned to (*Z,E*)-9,12-14:OAc

Former studies on *S. littoralis* (Ljungberg et al., 1993; Quero et al., 1996) identified one pheromone-sensitive OSN type tuned to (*Z,E*)-9,12-14:OAc in type 2 long trichoid sensilla (here referred as the LT2A OSN type). The response spectrum of this OSN type matches that of SlitOR6, but no OSN detecting both (*Z,E*)-9,12-14:OAc and (Z)9-14:OAc, which would correspond to SlitOR13 response profile, was found. Considering that those electrophysiology studies focused on sensilla located on the proximal part of the antennae, where trichoid sensilla are abundant and easy to record, we investigated whether OSNs with a response profile matching that of SlitOR13 could be found on more distal parts of the antennae. We thus performed single-sensillum recordings all along the antennae, using the panel of 26 pheromone compounds previously used for the functional characterization of SlitORs. We found the previously described trichoid sensillum types LT1 and LT2 in both the proximal and

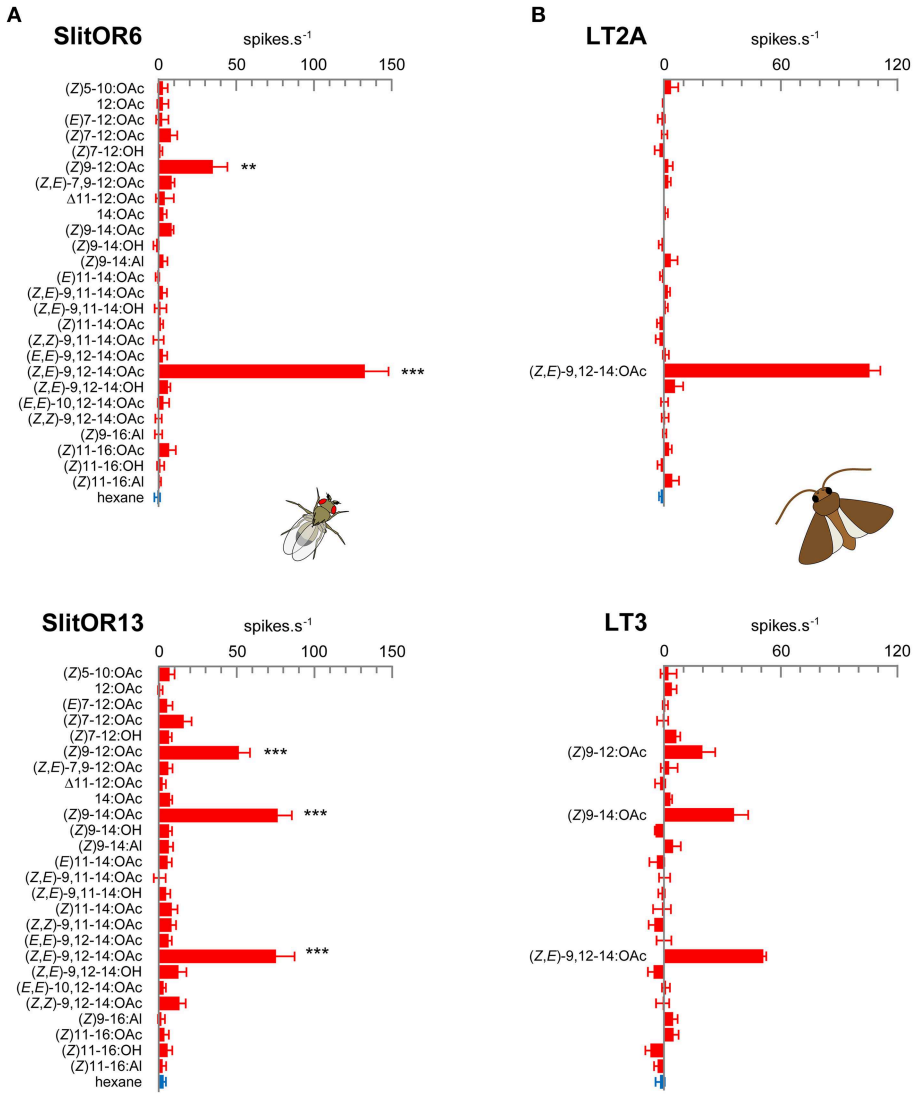

FIGURE 1 | (A) Response spectra of SlitOR6 and SlitOR13 expressed in *Drosophila* at1 OSNs. **(B)** Response spectra of LT2A and LT3 OSNs from *S. littoralis* male antennae. Values correspond to the increase in the frequency of action potentials emitted by the OSN during the odorant stimulation. The stimulus panel consisted in 26 pheromone compounds (10 μg in the stimulus cartridge for single-sensillum recordings on *Drosophila*, 1 μg for recordings on *Spodoptera*). Error bars indicate SEM ($n = 5$–9 for SlitOR6, $n = 7$–12 for SlitOR13, and $n = 3$ for *S. littoralis* OSNs). ***$P < 0.001$, **$P < 0.01$ significantly different from the response to solvent (Mann-Whitney *U*-test).

distal part of the antennae, and LT2A OSNs displayed responses only to (*Z*,*E*)-9,12-14:OAc (106 spikes.s^{-1}), thus confirming their specificity toward this compound (**Figure 1B**). Besides that, we identified a new functional type of long trichoid sensilla on the distal part of the antennae only, further referred as LT3. A cross-adaptation experiment determined that these sensilla housed only one OSN (data not shown). LT3 OSNs responded to (*Z*,*E*)-9,12-14:OAc (51 spikes.s^{-1}), (*Z*)9-14:OAc (36 spikes.s^{-1}) and to a lesser extent to (*Z*)9-12:OAc (20 spikes.s^{-1}). The low number of recordings (LT3 were scarce and only three could be registered) did not allow statistical analysis. The LT2A and LT3 OSN detection spectra thus clearly matched those of *Drosophila* OSNs expressing SlitOR6 and SlitOR3, respectively (**Figures 1A,B**).

Expression Profiles of SlitOR6 and SlitOR13

We next verified whether expression profiles of SlitOR6 and 13 along the antennae would correlate with the localization of LT2 and LT3 sensilla. We used quantitative real-time PCR to compare the relative expression levels of the two PRs in the proximal and distal parts of male antennae (**Figure 3**). We found that SlitOR6 was expressed in both parts, with a two-fold enrichment in the proximal part. By contrast, SlitOR13 expression was found only in the distal part of the antennae, where LT3 were localized.

Phylogenetic Analysis of Pheromone Receptors

In order to gather information about the evolutionary history of SlitOR6 and 13, we built a maximum-likelihood phylogeny of

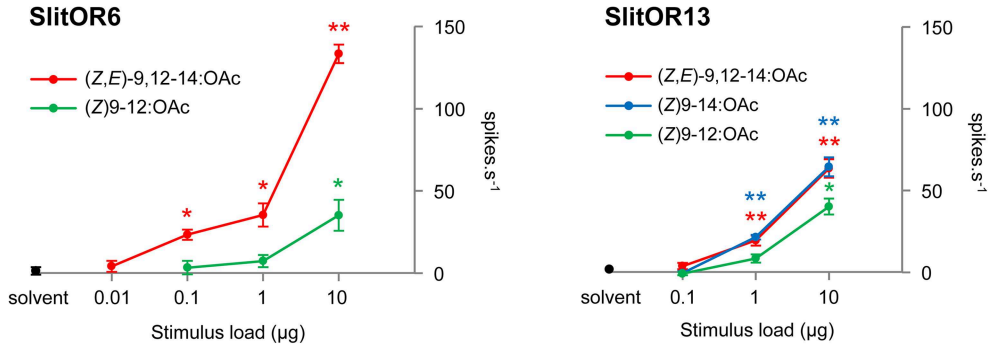

FIGURE 2 | Dose-response experiments on SlitOR6 and SlitOR13 expressed in *Drosophila* at1 OSNs. Responses to previously identified ligands have been tested at doses from 10 µg down to 0.1 or 0.01 µg in the stimulus cartridge. Error bars indicate SEM ($n = 5$). $^{**}P < 0.001$, $^{*}P < 0.01$ significantly different from the response to solvent (Mann-Whitney *U*-test).

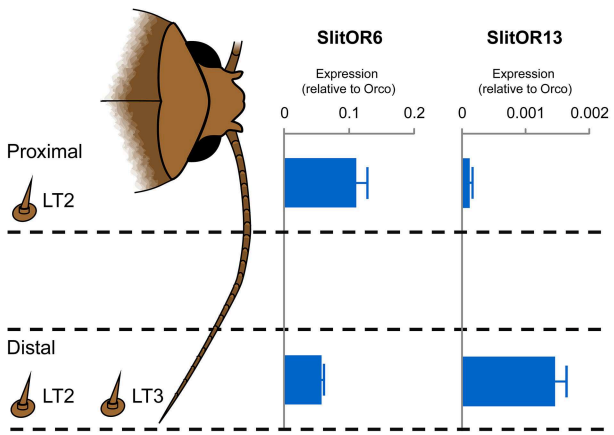

FIGURE 3 | Relative expression levels of SlitOR6 and SlitOR13 in the proximal and the distal parts of the *S. littoralis* male antennae. Expression levels have been normalized to the expression of the obligate olfactory co-receptor ORco. The occurrence of the LT2 and LT3 trichoid sensilla in the proximal and distal parts is also indicated. Error bars indicate standard deviation.

Lepidoptera candidate PRs, including 147 amino acid sequences from 37 species of moths and butterflies (Supplementary Table S2). These receptors grouped within five different paralogous lineages supported by the likelihood-ratio test, each lineage containing sequences from a various number of Lepidoptera super-families (**Figure 4** and Supplementary Figure S1). In the Noctuoidea super-family, numerous gene duplications occurred in the lineage E and account for the large number of PRs observed in these species (**Figure 4** and Supplementary Figure S1).

The two *S. littoralis* receptors under scrutiny here, SlitOR6 and 13, belong to the paralogous lineages D and E, respectively. Apart from closely related orthologs in *S. exigua* and *S. litura*, no other PRs with similar functional properties have been found in these lineages. However, the receptor PxylOR4 from the diamondback moth *Plutella xylostella* (sub-family Yponeumotoidea) also binds (*Z*,*E*)-9,12-14:OAc, and belongs to the B lineage. This indicates that the ability to bind (*Z*,*E*)-9,12-14:OAc emerged three times independently, in three distinct paralogous lineages.

Amino Acid Sites under Positive Selection

Next, we searched for regions in *Spodoptera* spp. OR6 and OR13 that could be responsible for the functional divergence of these receptors. We carried out a large-scale analysis of selective pressures acting on Lepidoptera PR genes using fastcodeml (Valle et al., 2014), a software implementation of branch-site probabilistic models of evolution that were introduced in (Yang and Nielsen, 2002). We found evidence for positive selection in numerous branches of the PR phylogeny, notably in the one leading to the pair of orthologs SlitOR6/SlituOR6, which both detect (*Z*,*E*)-9,12-14:OAc (**Figure 5A**), and in the one leading to *Spodoptera* OR13 orthologs, detecting (*Z*,*E*)-9,12-14:OAc and (*Z*)9-14:OAc (**Figure 5C**). We then mapped the candidate sites under positive selection in those two branches, as inferred by the Bayes empirical Bayes approach, on the predicted topology of *Spodoptera* OR6 (**Figure 5B**) and OR13 (**Figure 5D**). For OR6, the four candidate sites were located within transmembrane domains (TM4 and TM5) as well as intra-cellular loops (IL2 and IL3). For OR13, six candidate sites were found, although with moderate support, in the intracellular N-terminus and in the IL1, EL1, TM4, and TM6 domains.

We then compared the localization of candidate positively selected sites in OR6 and OR13 with those found in other branches of the PR phylogeny. More than 80% of these sites were found in transmembrane domains, notably TM4, and intra-cellular loops, notably IL1 and IL2 (**Figure 5E**). This is fully consistent with what we have found for OR6, whereas our analysis for OR13 highlighted sites that fell outside of these common regions of positive selection.

Discussion

Moth PRs are often cited as striking examples of the sensitivity and specificity that can be ensured by insect ORs. However, most studies used a small panel of pheromone compounds for stimulation, usually restricted to those found in the species

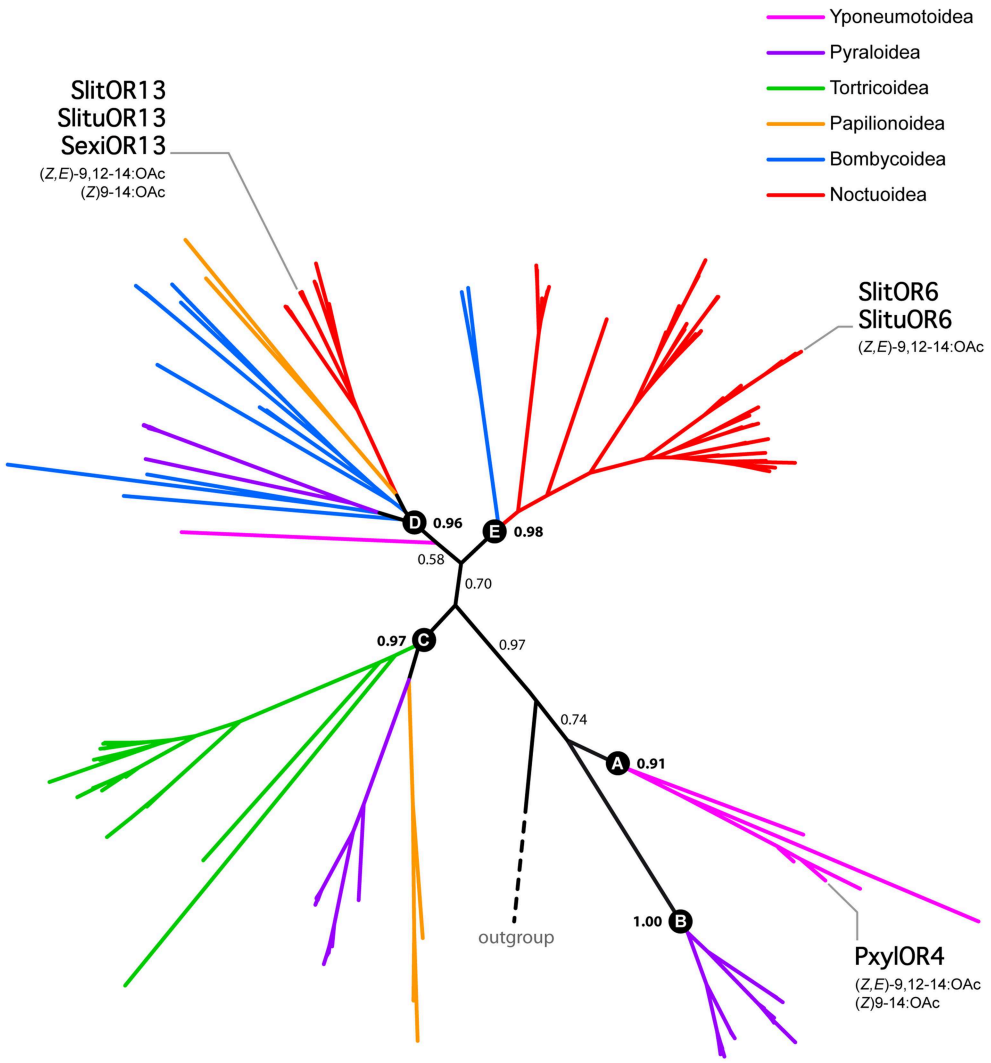

FIGURE 4 | Unrooted maximum likelihood tree of Lepidoptera candidate PRs, highlighting the phylogenetic position of receptors tuned to (*Z*,*E*)-9,12-14:OAc. Branches are color-coded following the Lepidoptera super-families. Letters from A to E correspond to different paralogous lineages. Support values correspond to the result of the likelihood-ratio test.

blend (Sakurai et al., 2004; Forstner et al., 2009) and at best in closely relatives (Mitsuno et al., 2008; Liu et al., 2013a,b; Zhang et al., 2015). Here, we have used a large panel of 26 compounds to functionally characterize four candidate PRs in *S. littoralis*. While SlitOR16 could not be expressed in *Drosophila* OSNs, thus precluding its functional characterization, its orthologs in *S. litura* and *S. exigua* bind preferentially (*Z*)9-14:OH (Liu et al., 2013a; Zhang et al., 2015). We can reasonably expect that SlitOR16 shares the same response spectrum. SlitOR11 could be expressed in *Drosophila* but remained silent, even after stimulation with such a large panel. Neither could OR11 orthologs be deorphanized in previous studies carried out in other noctuid species, including *S. exigua* and *S. litura* (Wang et al., 2011; Liu et al., 2013a,b; Jiang et al., 2014; Zhang et al., 2015). In *D. melanogaster*, it has been observed that OR67d-expressing neurons need the pheromone-binding protein (PBP) LUSH to detect its pheromone ligand (Ha and Smith, 2006). It could be hypothesized that SlitOR11 and its orthologs need a specific PBP to detect pheromone compounds. Another possible explanation is that OR11 is in fact not a PR, although it groups within the candidate PR clade in the Lepidoptera OR phylogeny (de Fouchier et al., 2014). In line with this hypothesis, it has been demonstrated that this PR clade actually includes receptors to plant odorants, such as the pear ester receptor of *Cydia pomonella* (Bengtsson et al., 2014).

The ligands we identified for SlitOR6 and SlitOR13 are the same as those previously identified for orthologous receptors in the closely related species, *S. exigua* and *S. litura*, although *S. exigua* OR6 could not be deorphanized (Liu et al., 2013a; Zhang et al., 2015). In addition, the larger panel used here (only 5 and 7 compounds were tested on *S. exigua* and *S. litura* ORs, respectively), confirms the narrow tuning of these receptors. Both

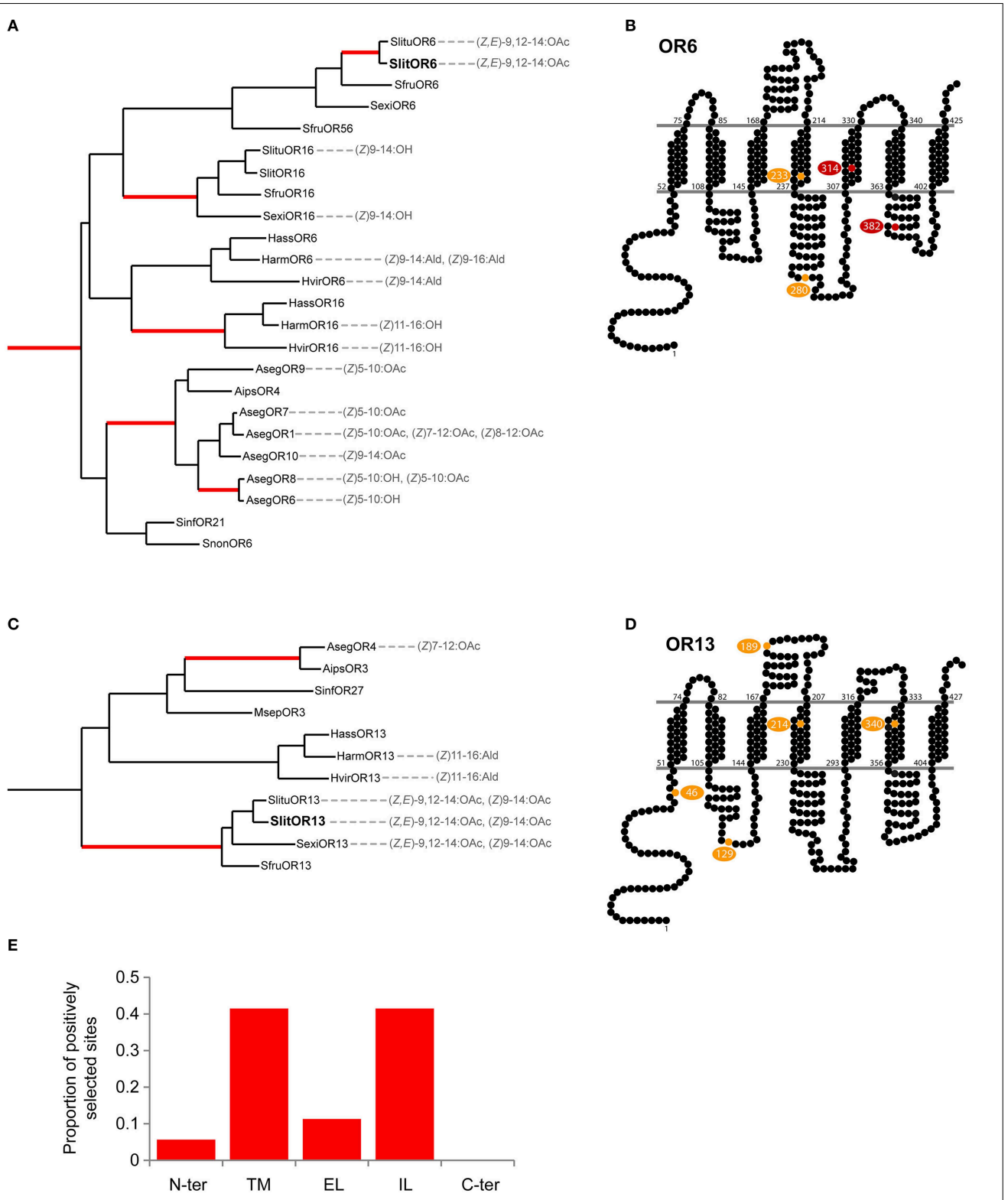

FIGURE 5 | Analysis of positive selection acting on PR genes. **(A,C)** Detail of clades containing *Spodoptera* OR6 and OR13 orthologs, showing branches (in red) in which there is evidence of positive selection. Ligands indicated are the best ligands found during the functional characterization of PRs (Wang et al., 2011; Liu et al., 2013a,b; Zhang and Lofstedt, 2013; Zhang et al., 2015). **(B,D)** Putative positively selected sites in *Spodoptera* OR6 and OR13 orthologs mapped onto the predicted topologies (yellow, posterior probability > 0.5; red, *PP* > 0.9). **(E)** Distribution of the candidate positively selected sites found in Lepidoptera PRs by type of protein domains: N-ter, N-terminus; TM, Trans-membrane domains; EL, Extracellular loops; IL, Intracellular loops; and C-ter, C-terminus.

SlitOR6 and SlitOR13 responded to the same minor component of the *S. littoralis* pheromone blend, (*Z*,*E*)-9,12-14:OAc, but with striking differences. Whereas SlitOR6 was highly specific and very sensitive, SlitOR13 detected (*Z*,*E*)-9,12-14:OAc, yet with less sensitivity than SlitOR6, and (*Z*)9-14:OAc, another minor component.

Using a combination of single-sensillum recordings along *S. littoralis* antennae and quantitative PCR, we could correlate the response spectra and the spatial distribution of SlitOR6 and OR13 with that of LT2A and LT3 OSNs, the latter type being described here for the first time. The difference observed between the responses of SlitOR6-expressing OSNs and LT2A OSNs to (*Z*)9-12:OAc could arise from the differences in the stimulus load (10 and 1 μg, respectively). Accordingly, SlitOR6 was not activated by lower amounts of (*Z*)9-12:OAc in dose-response experiments. We thus propose that SlitOR6 is expressed in the LT2A OSNs and that SlitOR13 is expressed in the newly described LT3 OSNs. The identification of a new type of pheromone-sensitive sensilla in *S. littoralis*, even after decades of electrophysiological studies (Muñoz et al., 2008; Binyameen et al., 2012), emphasizes the benefits of studying in detail both response spectra and expression patterns of ORs. Albeit difficult to perform, precise single-sensillum recordings at the distal part of the antennae deserve future attention and may lead to the identification of new functional types of sensilla. Whereas sucrose-detecting gustatory sensory neurons located in chaetic sensilla have been found to be especially abundant at the tip of *Helicoverpa armigera* antennae (Jørgensen et al., 2007), we report here for the first time such a specific localization of an OSN type. Whether such an atypical spatial repartition is important for moth orientation remains to be determined. In *H. armigera*, the relative abundance of sugar-detecting neurons in the distal region of the antenna has been linked with an ecological need to probe the nectar of the flowers (Jørgensen et al., 2007). In female *S. littoralis*, it has been hypothesized that the lowered response sensitivity of distal OSNs to several, but not all, odorants could participate in close range orientation to high concentration odor flux (Binyameen et al., 2012). Similarly, one can speculate that the distal localization of SlitOR13-expressing OSNs may play a role in a shorter range assessment of the pheromone blend composition, during the mating process.

The functional redundancy of PRs tuned to minor components could provide a mean for the precise coding of ratios within the pheromone blend. In *S. littoralis*, (*Z*,*E*)-9,12-14:OAc is present at a very low amount in the blend, and is even lacking in some strains (Muñoz et al., 2008). However, when added at a 1% ratio to the major pheromone component (*Z*,*E*)-9,11-14:OAc, it has a synergistic effect on the attraction of males, and increasing the ratio of (*Z*,*E*)-9,12-14:OAc in the blend drastically reduces male capture in pheromone traps (Kehat et al., 1976; Campion et al., 1980). Minute amounts of (*Z*,*E*)-9,12-14:OAc may enhance the attraction of males toward (*Z*,*E*)-9,11-14:OAc through the activation of the LT2A neurons (expressing the very sensitive SlitOR6). With increasing doses of (*Z*,*E*)-9,12-14:OAc, the LT3 OSN population (expressing the less sensitive SlitOR13) may also be recruited and would inhibit the attractive behavior triggered by (*Z*,*E*)-9,11-14:OAc. The antagonistic effect of high doses of (*Z*)9-14:OAc, the other ligand of SlitOR13, observed in field trapping studies (Campion et al., 1980) lends further support to this model. In nature, this precise detection of high doses of (*Z*,*E*)-9,12-14:OAc by *S. littoralis* males may constitute a mechanism to avoid interbreeding with the sympatric species *S. exigua*, whose sex pheromone contains high amounts of (*Z*,*E*)-9,12-14:OAc (Acin et al., 2010).

Focusing on OR13, it is worth noticing that this receptor exhibits different sensitivities toward its two ligands in the three *Spodoptera* species. Whereas SexiOR13 is more sensitive to (*Z*,*E*)-9,12-14:OAc than to (*Z*)9-14:OAc (Liu et al., 2013a), the opposite is observed for SlituOR13 (Zhang et al., 2015) and here SlitOR13 had the same sensitivity for both pheromone components. Such differences may be relevant for an efficient coding of ratios in the different *Spodoptera* species.

Even though the four candidate PRs have been studied in three different *Spodoptera* species (Liu et al., 2013a; Zhang et al., 2015; this study), no receptor has been identified for (*Z*,*E*)-9,11-14:OAc, the major pheromone component in *S. littoralis* and *S. litura* (Muñoz et al., 2008; Saveer et al., 2014). In a similar way, only two minor components of the *S. littoralis* pheromone blend, (*Z*)9-14:OAc and (*Z*,*E*)-9,12-14:OAc, could be assigned to a receptor, leaving all the other minor components orphans. This suggests that more PRs await further identification and/or that PBPs could modify *Spodoptera* PR response spectra as observed in *P. xylostella* (Sun et al., 2013).

As revealed by our phylogenetic analysis, the ability to bind (*Z*,*E*)-9,12-14:OAc appeared independently in three paralogous lineages. This prompted us to investigate whether similar evolutionary mechanisms occurred in each case. We detected episodes of positive selection in numerous branches of the PR phylogeny, and the putative positively selected sites were located mainly in TM and IL domains, which is consistent with studies on other insects. In ORs of the pea aphid, most positively selected sites have been found in TM domains (Smadja et al., 2009) and in *Drosophila*, such sites have been identified mostly in the IL domains (Guo and Kim, 2007). We found sites under positive selection in *Spodoptera* OR6 and OR13 orthologs, but no shared sites. This suggests that the capacity to bind (*Z*,*E*)-9,12-14:OAc emerged either due to positive selection acting on different amino acids in the two lineages or due to genetic drift rather than positive selection. As in the majority of other PRs, candidate positively selected sites in OR6 (and to a lesser extent in OR13) were located in TM and IL domains. What could be the functional significance of modifications occurring in these domains? Molecular modeling of the interactions between *Drosophila* ORs and their cognate ligands led to the hypothesis that the ligand-binding pocket is located on the extracellular sides of TM domains (Guo and Kim, 2010). There are experimental evidences that mutations in TM2, 3 and 4 domains affect ligand binding in *Drosophila*, mosquito or moth receptors (Nichols and Luetje, 2010; Pellegrino et al., 2011; Leary et al., 2012; Hughes et al., 2014) whereas mutations in TM5, 6 and 7 affect the ion channel function of a moth PR (Nakagawa et al., 2012). An *in silico* analysis of insect OR structural features also identified N-ter, EL2 and IL3 domains as the most evolutionary constrained (Hopf et al., 2015), which is consistent with their

importance for the correct functioning of ORs (Benton et al., 2006; Jin et al., 2008; Xu and Leal, 2013). Further experimental validations are needed to confirm whether positive selection has indeed been a driving force for the diversification of moth PRs or not. This will shed light on the evolutionary mechanisms at the base of the evolution of pheromone communication and reproductive isolation in this major insect group.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2015.00095

References

Abascal, F., Zardoya, R., and Posada, D. (2005). ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* 21, 2104–2105. doi: 10.1093/bioinformatics/bti263

Acin, P., Rosell, G., Guerrero, A., and Quero, C. (2010). Sex pheromone of the spanish population of the beet armyworm *Spodoptera exigua*. *J. Chem. Ecol.* 36, 778–786. doi: 10.1007/s10886-010-9817-z

Anisimova, M., and Gascuel, O. (2006). Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. *Syst. Biol.* 55, 539–552. doi: 10.1080/10635150600755453

Bengtsson, J. M., Gonzalez, F., Cattaneo, A. M., Montagné, N., Walker, W. B., Bengtsson, M., et al. (2014). A predicted sex pheromone receptor of codling moth *Cydia pomonella* detects the plant volatile pear ester. *Front. Ecol. Evol.* 2:33. doi: 10.3389/fevo.2014.00033

Bengtsson, J. M., Trona, F., Montagné, N., Anfora, G., Ignell, R., Witzgall, P., et al. (2012). Putative chemosensory receptors of the codling moth, *Cydia pomonella*, identified by antennal transcriptome analysis. *PLoS ONE* 7:e31620. doi: 10.1371/journal.pone.0031620

Benton, R., Sachse, S., Michnick, S. W., and Vosshall, L. B. (2006). Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors *in vivo*. *PLoS Biol.* 4:e20. doi: 10.1371/journal.pbio.0040020

Binyameen, M., Anderson, P., Ignell, R., Seada, M. A., Hansson, B. S., and Schlyter, F. (2012). Spatial organization of antennal olfactory sensory neurons in the female *Spodoptera littoralis* moth: differences in sensitivity and temporal characteristics. *Chem. Senses* 37, 613–629. doi: 10.1093/chemse/bjs043

Campion, D. G., Hunter-Jones, P., Mcveigh, L. J., Hall, D. R., Lester, R., and Nesbitt, B. F. (1980). Modification of the attractiveness of the primary pheromone component of the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), by secondary pheromone components and related chemicals. *Bull. Entomol. Res.* 70, 417. doi: 10.1017/s0007485300007689

Cardé, R. T., and Haynes, K. F. (2004). "Structure of the pheromone communication channel in moths," in *Advances in Insect Chemical Ecology*, eds R. T. Cardé and J. G. Millar (Cambridge: Cambridge University Press), 283–331.

de Bruyne, M., and Baker, T. C. (2008). Odor detection in insects: volatile codes. *J. Chem. Ecol.* 34, 882–897. doi: 10.1007/s10886-008-9485-4

de Fouchier, A., Montagné, N., Mirabeau, O., and Jacquin-Joly, E. (2014). "Current views on the function and evolution of olfactory receptors in Lepidoptera," in *Short Views on Insect Biochemistry and Molecular Biology*, eds R. Chandrasekar, B. K. Tyagi, Z. Z. Gui, and G. R. Reeck (Manhattan, KS: International Book Mission), 385–408.

Engsontia, P., Sangket, U., Chotigeat, W., and Satasook, C. (2014). Molecular evolution of the odorant and gustatory receptor genes in lepidopteran insects: implications for their adaptation and speciation. *J. Mol. Evol.* 79, 21–39. doi: 10.1007/s00239-014-9633-0

Forstner, M., Breer, H., and Krieger, J. (2009). A receptor and binding protein interplay in the detection of a distinct pheromone component in the silkmoth *Antheraea polyphemus*. *Int. J. Biol. Sci.* 5, 745–757. doi: 10.7150/ijbs.5.745

Grosse-Wilde, E., Kuebler, L. S., Bucks, S., Vogel, H., Wicher, D., and Hansson, B. S. (2011). Antennal transcriptome of *Manduca sexta*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 7449–7454. doi: 10.1073/pnas.1017963108

Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* 59, 307–321. doi: 10.1093/sysbio/syq010

Guo, S., and Kim, J. (2007). Molecular evolution of *Drosophila* odorant receptor genes. *Mol. Biol. Evol.* 24, 1198–1207. doi: 10.1093/molbev/msm038

Guo, S., and Kim, J. (2010). Dissecting the molecular mechanism of drosophila odorant receptors through activity modeling and comparative analysis. *Proteins* 78, 381–399. doi: 10.1002/prot.22556

Ha, T. S., and Smith, D. P. (2006). A pheromone receptor mediates 11-cis-vaccenyl acetate-induced responses in *Drosophila*. *J. Neurosci.* 26, 8727–8733. doi: 10.1523/JNEUROSCI.0876-06.2006

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. doi: 10.1038/ncomms7077

Hughes, D. T., Wang, G., Zwiebel, L. J., and Luetje, C. W. (2014). A determinant of odorant specificity is located at the extracellular loop 2-transmembrane domain 4 interface of an *Anopheles gambiae* odorant receptor subunit. *Chem. Senses* 39, 761–769. doi: 10.1093/chemse/bju048

Jiang, X. J., Guo, H., Di, C., Yu, S., Zhu, L., Huang, L. Q., et al. (2014). Sequence similarity and functional comparisons of pheromone receptor orthologs in two closely related *Helicoverpa* species. *Insect. Biochem. Mol. Biol.* 48, 63–74. doi: 10.1016/j.ibmb.2014.02.010

Jin, X., Ha, T. S., and Smith, D. P. (2008). SNMP is a signaling component required for pheromone sensitivity in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10996–11001. doi: 10.1073/pnas.0803309105

Jones, D. T., Taylor, W. R., and Thornton, J. M. (1992). The rapid generation of mutation data matrices from protein sequences. *Comput. Appl. Biosci.* 8, 275–282.

Jordan, M. D., Anderson, A., Begum, D., Carraher, C., Authier, A., Marshall, S. D., et al. (2009). Odorant receptors from the light brown apple moth (*Epiphyas postvittana*) recognize important volatile compounds produced by plants. *Chem. Senses* 34, 383–394. doi: 10.1093/chemse/bjp010

Jorgensen, K., Almaas, T. J., Marion-Poll, F., and Mustaparta, H. (2007). Electrophysiological characterization of responses from gustatory receptor neurons of sensilla chaetica in the moth *Heliothis virescens*. *Chem. Senses* 32, 863–879. doi: 10.1093/chemse/bjm057

Kaissling, K. E. (1996). Peripheral mechanisms of pheromone reception in moths. *Chem. Senses* 21, 257–268.

Katoh, K., Kuma, K., Toh, H., and Miyata, T. (2005). MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Res.* 33, 511–518. doi: 10.1093/nar/gki198

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Kehat, M., Greenberg, S., and Tamaki, Y. (1976). Field evaluation of the synthetic sex pheromone, as an attractant for males of the cotton leafworm,

Spodoptera littoralis (Boisd.), in Israel. *Appl. Entomol. Zool.* 11, 45–52. doi: 10.1303/aez.11.45

Krieger, J., Grosse-Wilde, E., Gohl, T., Dewer, Y. M., Raming, K., and Breer, H. (2004). Genes encoding candidate pheromone receptors in a moth (*Heliothis virescens*). *Proc. Natl. Acad. Sci. U.S.A.* 101, 11845–11850. doi: 10.1073/pnas.0403052101

Kurtovic, A., Widmer, A., and Dickson, B. J. (2007). A single class of olfactory neurons mediates behavioural responses to a *Drosophila* sex pheromone. *Nature* 446, 542–546. doi: 10.1038/nature05672

Larsson, M. C., Domingos, A. I., Jones, W. D., Chiappe, M. E., Amrein, H., and Vosshall, L. B. (2004). Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* 43, 703–714. doi: 10.1016/j.neuron.2004.08.019

Leary, G. P., Allen, J. E., Bunger, P. L., Luginbill, J. B., Linn, C. E. Jr., Macallister, I. E., et al. (2012). Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14081–14086. doi: 10.1073/pnas.1204661109

Legeai, F., Malpel, S., Montagné, N., Monsempes, C., Cousserans, F., Merlin, C., et al. (2011). An Expressed Sequence Tag collection from the male antennae of the Noctuid moth *Spodoptera littoralis*: a resource for olfactory and pheromone detection research. *BMC Genomics* 12:86. doi: 10.1186/1471-2164-12-86

Letunic, I., and Bork, P. (2011). Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res.* 39, W475–W478. doi: 10.1093/nar/gkr201

Liu, C., Liu, Y., Walker, W. B., Dong, S., and Wang, G. (2013a). Identification and functional characterization of sex pheromone receptors in beet armyworm *Spodoptera exigua* (Hubner). *Insect. Biochem. Mol. Biol.* 43, 747–754. doi: 10.1016/j.ibmb.2013.05.009

Liu, Y., Liu, C., Lin, K., and Wang, G. (2013b). Functional specificity of sex pheromone receptors in the cotton bollworm *Helicoverpa armigera*. *PLoS ONE* 8:e62094. doi: 10.1371/journal.pone.0062094

Ljungberg, H., Anderson, P., and Hansson, B. S. (1993). Physiology and morphology of pheromone-specific sensilla on the antennae of male and female *Spodoptera littoralis* (Lepidoptera, Noctuidae). *J. Insect Physiol.* 39, 253–260. doi: 10.1016/0022-1910(93)90096-A

Mitsuno, H., Sakurai, T., Murai, M., Yasuda, T., Kugimiya, S., Ozawa, R., et al. (2008). Identification of receptors of main sex-pheromone components of three Lepidopteran species. *Eur. J. Neurosci.* 28, 893–902. doi: 10.1111/j.1460-9568.2008.06429.x

Montagné, N., Chertemps, T., Brigaud, I., Francois, A., Francois, M. C., de Fouchier, A., et al. (2012). Functional characterization of a sex pheromone receptor in the pest moth *Spodoptera littoralis* by heterologous expression in *Drosophila*. *Eur. J. Neurosci.* 36, 2588–2596. doi: 10.1111/j.1460-9568.2012.08183.x

Muñoz, L., Rosell, G., Quero, C., and Guerrero, A. (2008). Biosynthetic pathways of the pheromone of the Egyptian armyworm *Spodoptera littoralis*. *Physiol. Entomol.* 33, 275–290. doi: 10.1111/j.1365-3032.2008.00645.x

Nakagawa, T., Pellegrino, M., Sato, K., Vosshall, L. B., and Touhara, K. (2012). Amino acid residues contributing to function of the heteromeric insect olfactory receptor complex. *PLoS ONE* 7:e32372. doi: 10.1371/journal.pone.0032372

Nakagawa, T., Sakurai, T., Nishioka, T., and Touhara, K. (2005). Insect sex-pheromone signals mediated by specific combinations of olfactory receptors. *Science* 307, 1638–1642. doi: 10.1126/science.1106267

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. doi: 10.1074/jbc.M109.058321

Pellegrino, M., Steinbach, N., Stensmyr, M. C., Hansson, B. S., and Vosshall, L. B. (2011). A natural polymorphism alters odour and DEET sensitivity in an insect odorant receptor. *Nature* 478, 511–514. doi: 10.1038/nature10438

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45. doi: 10.1093/nar/29.9.e45

Poitout, S., and Buès, R. (1974). Elevage de chenilles de vingt-huit espèces de Lépidoptères Noctuidae et de deux espèces d'Arctiidae sur milieu artificiel simple. Particularités de l'élevage selon les espèces. *Ann. Zool. Ecol. Anim.* 6, 431–441.

Quero, C., Lucas, P., Renou, M., and Guerrero, A. (1996). Behavioral responses of *Spodoptera littoralis* males to sex pheromone components and virgin females in wind tunnel. *J. Chem. Ecol.* 22, 1087–1102. doi: 10.1007/Bf02027947

Sakurai, T., Nakagawa, T., Mitsuno, H., Mori, H., Endo, Y., Tanoue, S., et al. (2004). Identification and functional characterization of a sex pheromone receptor in the silkmoth *Bombyx mori*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16653–16658. doi: 10.1073/pnas.0407596101

Salama, H. S., Dimetry, N. Z., and Salem, S. A. (1971). On the host preference and biology of the cotton leaf worm *Spodoptera littoralis* Bois. *Z Angew. Entomol.* 67, 261–266. doi: 10.1111/j.1439-0418.1971.tb02122.x

Saveer, A. M., Becher, P. G., Birgersson, G. R., Hansson, B. S., Witzgall, P., and Bengtsson, M. (2014). Mate recognition and reproductive isolation in the sibling species *Spodoptera littoralis* and *Spodoptera litura*. *Front. Ecol. Evol.* 2:18. doi: 10.3389/fevo.2014.00018

Shimodaira, H. (2002). An approximately unbiased test of phylogenetic tree selection. *Syst. Biol.* 51, 492–508. doi: 10.1080/10635150290069913

Smadja, C., Shi, P., Butlin, R. K., and Robertson, H. M. (2009). Large gene family expansions and adaptive evolution for odorant and gustatory receptors in the pea aphid, *Acyrthosiphon pisum*. *Mol. Biol. Evol.* 26, 2073–2086. doi: 10.1093/molbev/msp116

Steinwender, B., Thrimawithana, A. H., Crowhurst, R. N., and Newcomb, R. D. (2015). Pheromone receptor evolution in the cryptic leafroller species, *Ctenopseustis obliquana* and *C. herana*. *J. Mol. Evol.* 80, 42–56. doi: 10.1007/s00239-014-9650-z

Sun, M., Liu, Y., Walker, W. B., Liu, C., Lin, K., Gu, S., et al. (2013). Identification and characterization of pheromone receptors and interplay between receptors and pheromone binding proteins in the diamondback moth, *Plutella xyllostella*. *PLoS ONE* 8:e62098. doi: 10.1371/journal.pone.0062098

Syed, Z., Kopp, A., Kimbrell, D. A., and Leal, W. S. (2010). Bombykol receptors in the silkworm moth and the fruit fly. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9436–9439. doi: 10.1073/pnas.1003881107

Valle, M., Schabauer, H., Pacher, C., Stockinger, H., Stamatakis, A., Robinson-Rechavi, M., et al. (2014). Optimization strategies for fast detection of positive selection on phylogenetic trees. *Bioinformatics* 30, 1129–1137. doi: 10.1093/bioinformatics/btt760

Wang, G., Vásquez, G. M., Schal, C., Zwiebel, L. J., and Gould, F. (2011). Functional characterization of pheromone receptors in the tobacco budworm *Heliothis virescens*. *Insect. Mol. Biol.* 20, 125–133. doi: 10.1111/j.1365-2583.2010.01045.x

Wanner, K. W., Nichols, A. S., Allen, J. E., Bunger, P. L., Garczynski, S. F., Linn, C. E., et al. (2010). Sex pheromone receptor specificity in the European corn borer moth, *Ostrinia nubilalis*. *PLoS ONE* 5:e8685. doi: 10.1371/journal.pone.0008685

Xu, P., and Leal, W. S. (2013). Probing insect odorant receptors with their cognate ligands: insights into structural features. *Biochem. Biophys. Res. Commun.* 435, 477–482. doi: 10.1016/j.bbrc.2013.05.015

Yang, Z., and dos Reis, M. (2011). Statistical properties of the branch-site test of positive selection. *Mol. Biol. Evol.* 28, 1217–1228. doi: 10.1093/molbev/msq303

Yang, Z., and Nielsen, R. (2002). Codon-substitution models for detecting molecular adaptation at individual sites along specific lineages. *Mol. Biol. Evol.* 19, 908–917. doi: 10.1093/oxfordjournals.molbev.a004148

Zhang, D. D., and Löfstedt, C. (2013). Functional evolution of a multigene family: orthologous and paralogous pheromone receptor genes in the turnip moth, *Agrotis segetum*. *PLoS ONE* 8, e77345. doi: 10.1371/journal.pone.0077345

Zhang, J., Yan, S., Liu, Y., Jacquin-Joly, E., Dong, S., and Wang, G. (2015). Identification and functional characterization of sex pheromone receptors in the common cutworm (*Spodoptera litura*). *Chem. Senses* 40, 7–16. doi: 10.1093/chemse/bju052

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A predicted sex pheromone receptor of codling moth *Cydia pomonella* detects the plant volatile pear ester

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Plant volatiles mediate host discrimination and host finding in phytophagous insects. Understanding how insects recognize these signals is a current challenge in chemical ecology research. Pear ester, ethyl (*E,Z*)-2,4-decadienoate, is a powerful, bisexual attractant of codling moth *Cydia pomonella* (Lepidoptera, Tortricidae) and strongly synergizes the male response to female-produced sex pheromone. We show here that the codling moth odorant receptor (OR) CpomOR3 is dedicated to detecting this plant volatile. Heterologous expression of CpomOR3 in *Drosophila* T1 trichoid and ab3A basiconic sensilla, followed by a screening with codling moth pheromone compounds and known plant volatile attractants, confirms that CpomOR3 binds to pear ester. Although CpomOR3 does not respond to any of the pheromone components tested, a phylogenetic analysis of lepidopteran chemosensory receptor genes reveals a close relationship of CpomOR3 with pheromone receptors (PRs) in moths. This corroborates the interaction of ecological and social chemosensory cues during premating communication. The finding that a plant volatile compound, pear ester, is a specific ligand for a PR-like lepidopteran receptor adds to our understanding of insect-plant interactions and emphasizes the interaction of natural and sexual selection during the phylogenetic divergence of insect herbivores.

Keywords: olfaction, odorant receptor, heterologous expression, semiochemical, sex pheromone, plant volatile, insect control

INTRODUCTION

Interactions between plants and insects shape many terrestrial ecosystems, and the primary mode of communication between plants and insects is chemical. Plant volatile chemicals mediate recognition of adult food sites, adequate oviposition sites and larval host plants (Bruce and Pickett, 2011) and accordingly play a prominent role in premating reproductive isolation and phylogenetic diversification of insect herbivores (Dres and Mallet, 2002; Smadja and Butlin, 2009; Matsubayashi et al., 2010). Decoding the plant volatile signatures that enable insects to discriminate between host and non-host plants is a long-standing research challenge in chemical ecology (Dethier, 1947, 1982; Ehrlich and Raven, 1964).

The identification of behaviorally active plant volatiles is a delicate and tedious task since plants release a large suite of volatiles, with no apparent correlation between the relative abundance of these compounds and their behavioral role in associated insects. Moreover, a behavioral response is frequently elicited by compound blends, where single compounds can often be exchanged with no apparent loss of activity (Bengtsson et al., 2006; Tasin et al., 2006, 2010; Pinero et al., 2008; Riffell et al., 2009; Cha et al., 2011; Schmidt-Busser et al., 2011; Thoming and Knudsen, 2014). This makes it particularly difficult to determine which plant volatiles encode host finding in phytophagous insects. In comparison, the identification of insect sex pheromones is facilitated by the production of few compounds in dedicated glands in one sex, together with a strong, distinctive behavioral response in the other.

The larvae of codling moth, *Cydia pomonella* (Lepidoptera, Tortricidae), feed on apple, pear, and walnut. The main sex pheromone compound codlemone, (*E,E*)-8,10-dodecadien-1-ol, was identified long ago (Roelofs et al., 1971; Beroza et al., 1974), but it is still open to question which compounds evoke attraction of egg-laying codling females to the plant host. Plant odorants obviously account for host attraction in codling moth, and several compounds from apple fruit and foliage elicit a strong antennal response. However, these compounds produce only a rather weak behavioral response (Bengtsson et al., 2001; Coracini et al., 2004; Hern and Dorn, 2004; Witzgall et al., 2005).

The strongest known kairomonal attractant is a pear ester, ethyl (*E,Z*)-2,4-decadienoate (Jennings et al., 1964; Berger and Drawert, 1984; Willner et al., 2013), which attracts codling moth adult males and females, as well as larvae (Knight and Light, 2001; Light et al., 2001; Light and Knight, 2005). This makes pear ester a versatile tool for sustainable insect control. It is used to monitor the seasonal abundance of codling moth (Knight and Light,

2012; Knight et al., 2013), as well as to enhance population control by mating disruption, in blends with codlemone (Knight et al., 2012). More recently, a microencapsulated formulation of pear ester has been developed for disruption of larval orientation and host finding (Light and Beck, 2012; Knight and Light, 2013).

Pear ester has been identified by screening codling moth antennal response to a wide range of apple and pear volatiles, followed by field trapping (Light et al., 2001; Light and Knight, 2005). Its biological significance is, however, not entirely clear, since it is found mainly in pear and only in some apple cultivars (Jennings et al., 1964; Berger and Drawert, 1984; Willner et al., 2013). The association of codling moth with cultivated apple is, on the other hand, recent and the response to pear ester may stem from an evolutionarily ancient host plant of codling moth.

Given the difficulties associated with completely assessing the pool of plant volatiles produced by the various host plants of codling moth, it is sensible to also investigate the response of single odorant receptors (ORs), many of which are likely dedicated to the perception of plant volatiles. ORs interface insects with their odor environment by binding odorants, and are expressed in olfactory sensory neurons (OSNs), which transmit olfactory information to the brain. The number of ORs expressed on the antenna and their compound-specificity determines the range of odorants an insect can detect. General ORs are tuned to environmental odors including plant volatiles, while pheromone receptors (PRs), a male-biased receptor clade, respond mainly to sex pheromones (Jacquin-Joly and Merlin, 2004; Ihara et al., 2013; Leal, 2013).

An emerging technique, which is quickly becoming an integral part of the toolbox for identification of behaviorally relevant plant odorants, is the functional characterization ("deorphanization") of ORs, following expression in heterologous expression systems. The OR repertoire of *Drosophila* has been studied exhaustively (Hallem et al., 2004; Kreher et al., 2005; Hallem and Carlson, 2006) and current research aims at other insect groups. For moths, a number of ORs and PRs have been identified and functionally characterized, using various heterologous expression systems, including human embryonic kidney (HEK) cells (Grosse-Wilde et al., 2007), *Xenopus* oocytes (Sakurai et al., 2004; Nakagawa et al., 2005; Jiang et al., 2014), Sf9, a cell line derived from fall armyworm *Spodoptera frugiperda* ovaries (Jordan et al., 2009), and *Drosophila* OSNs (Syed et al., 2010; Montagné et al., 2012), which is an *in vivo* antennal expression approach.

Expressing ORs in single *Drosophila* neurons comprises two main advantages. The biochemical environment of *Drosophila* OSNs endogenously provides odorant binding proteins (OBPs) and Orco, a canonical receptor conserved across insects (Krieger et al., 2003; Jones et al., 2005; Leal, 2013), which may enhance response sensitivity and specificity of the expressed OR, compared with non-insect cell lines. In addition, electrophysiological techniques, namely single sensillum recordings (SSRs) are well established for *Drosophila* sensilla.

Two main systems are available for expression and deorphanization of ORs in *Drosophila* OSNs, the "empty neuron" (ab3A) in ab3 basiconic sensilla, which lacks its native OR (Dobritsa et al., 2003) and the $Or67d^{GAL4}$ knock-in mutant line in trichoid T1 sensilla (Kurtovic et al., 2007). While the empty neuron system has been used mainly to functionally characterize general odorant receptors, pheromone receptors may respond more strongly when expressed in T1 rather than in ab3A (Syed et al., 2010; Montagné et al., 2012).

We have previously identified 43 candidate OR protein sequences in the antennal transcriptome of codling moth, five of which cluster within the conserved pheromone receptor clade of lepidopteran PRs (Bengtsson et al., 2012).

We here show that CpomOR3, belonging to the PR clade, is strictly tuned to pear ester. This result emphasizes the biological significance of pear ester (Light et al., 2001) and shows that the PR clade contains co-evolving receptors for sex pheromones and for host odorants. This corroborates the modulation of male sexual behavior by host plant odorants in codling moth (Trona et al., 2010, 2013), and adds to our understanding of the evolution of sexual communication and olfaction-driven speciation in insect herbivores.

METHODS

INSECTS, DISSECTION, AND RNA EXTRACTION

Cydia pomonella pupae were obtained from a laboratory rearing center (Andermatt Biocontrol, Grossdietwil, Switzerland), and adults were allowed to emerge in cages kept at 23°C, 70 ± 5% relative humidity and a 16 h:8 h light:dark cycle, and fed with 10% sugar solution. For dissections, 2–3 day old female and male insects were used. Using sharp forceps, antennae were removed at the base of the pedicel, and legs at the coxa. For thorax samples, head, wings, legs, and abdomen were removed. Wings were removed at their base, and the abdomen removed at the connection to the thorax. All body parts were immediately flash-frozen using liquid nitrogen, and thereafter kept at −80°C. RNAs were extracted using the RNeasy kit (Qiagen, Hilden, Germany).

RAPID AMPLIFICATION OF cDNA ENDS (RACE)-PCR

RACE-PCR was performed to obtain the complete open reading frame (ORF) for *CpomOR3*. A cDNA library for extension in the 5′ direction was created using the SMARTer kit (Clontech, Mountain View, CA, USA) on male antennal RNA. For the PCR reaction, the Advantage 2 kit (Clontech) was used, with a temperature program of 95°C for 2 min, then 30 cycles of 95°C for 1 min, 65°C for 90 s, 68°C for 2 min and a final elongation of 68°C for 7 min. A gene-specific primer (5′-CCCTAGAGCTTCGGTGTCCAATGTAGAGC-3′) was used together with the Universal primer mix (Clontech). The PCR product was analyzed by electrophoresis on an agarose gel, and the relevant band excised and purified by the Gel extraction kit (Qiagen). It was then cloned into the pGEM®-T Easy plasmid (Promega, Fitchburg, WI, USA), with which TOP10 cells were transformed (Invitrogen, Life Technologies, Carlsbad, CA, USA). Plasmids were subsequently purified using the Miniprep kit (Qiagen). Purified plasmids were quantified by nanodrop (Nanodrop 8000 UV-Vis Spectrophotometer, Thermo Scientific, Wilmington, DE, USA) and then Sanger sequenced (3730xl Applied Biosystems, Life Technologies) using the forward and reverse M13 universal primers. Transmembrane domains were predicted using TMHMM 2.0 (http://www.cbs.dtu.dk/services/

TMHMM/), on sequence translated to protein using ExPASy (http://web.expasy.org/translate/).

PHYLOGENETIC ANALYSIS

Amino acid sequences of CpomORs clustering in the candidate PR clade (Bengtsson et al., 2012) were included in a dataset together with sequences of candidate PRs from the following Lepidoptera: *Antheraea polyphemus* (Forstner et al., 2009), *Bombyx mori* (Nakagawa et al., 2005), *Danaus plexippus* (Zhan et al., 2011), *Diaphania indica* (Mitsuno et al., 2008), *Epiphyas postvittana* (Jordan et al., 2009), *Heliconius melpomene* (Heliconius Genome Consortium, 2012), *Helicoverpa armigera* (Liu et al., 2012), *Heliothis virescens* (Grosse-Wilde et al., 2007; Wang et al., 2010), *Manduca sexta* (Grosse-Wilde et al., 2010), *Mythimna separata* (Mitsuno et al., 2008), *Ostrinia furnacalis* (Miura et al., 2010; Leary et al., 2012), *O. nubilalis* (Wanner et al., 2010; Leary et al., 2012), *O. scapulalis* (Miura et al., 2009, 2010), *Plutella xylostella* (Mitsuno et al., 2008; Sun et al., 2013), *Spodoptera exigua* (Liu et al., 2013) and *S. littoralis* (Legeai et al., 2011; Montagné et al., 2012). Sequences from *B. mori* (BmorOR6) and *H. melpomene* (HmelOR5, 6, and 7) were also included in the dataset as external groups, since they belong to the sister group to the PR clade (Poivet et al., 2013). The CpomOR1 sequence was not included in the dataset because of its short length (only 101 amino acid residues). The 74 amino acid sequences were aligned using the online version of MAFFT v.7 (Katoh and Standley, 2013), with the G-INS-i algorithm (Katoh et al., 2005) and default parameters.

Phylogenetic reconstruction was performed using the maximum likelihood method. The LG+I+G+F substitution model (Le and Gascuel, 2008) was determined as the best-fit model of protein evolution by ProtTest 2.4 (Abascal et al., 2005) following Akaike information criterion. Rate heterogeneity was set at four categories, and the gamma distribution parameter and the proportion of invariable sites were estimated from the dataset. Tree reconstruction was performed using PhyML 3.0 (Guindon et al., 2010), with both SPR (Subtree Pruning and Regrafting) and NNI (Nearest Neighbor Interchange) methods for tree topology improvement. Node support was estimated using a bootstrap procedure based on 100 replicates, and nodes supported by a bootstrap value below 70% were collapsed. The figure was created using the iTOL web server (Letunic and Bork, 2011) and Adobe Illustrator.

REVERSE TRANSCRIPTION (RT)-PCR FOR *CPOMOR3* EXPRESSION ANALYSIS

cDNAs were synthesized from RNAs extracted from different tissues using the RT-for-PCR kit (Clontech), following the recommended protocol. Integrity of cDNAs was tested by PCR, using degenerate primers for *RPL8* (Forward primer 5′-GAGTCATCCGAGCTCARMGNAARGG-3′; Reverse primer 5′-CCAGCAGTTTCGCTTNACYTTRTA-3′) and GoTaq Green Master Mix (Promega) with an annealing temperature of 54°C. PCR reactions to screen for expression of *CpomOR3* in different tissues used GoTaq Green Master Mix, and consisted of an initial 5-min step at 94°C, and then 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and a final 7-min step at 72°C. Gene specific primers (GSP) for *CpomOR3,* 5′-AGATGAAGAGTATCGGAATTGCATGG-3′ (forward) and 5′-CCAACTGGGATCATGCCACAAGC-3′ (reverse), were used, giving a product of 436 bp. Product identity was confirmed by direct sequencing, following gel extraction (QIAquick Gel Extraction Kit, Qiagen). Each PCR reaction was repeated three times and control consisted of a no template PCR. PCR was performed in parallel on *C. pomonella* genomic DNA templates, extracted from larvae using PureLink Genomic DNA kit (Invitrogen). No amplification or amplification of larger size bands was observed, revealing specific cDNA amplification at the expected size. Products were analyzed on a 1.5% agarose gel and visualized after staining with ethidium bromide using a Gel Doc XR (Bio-Rad, Hercules, CA, USA).

HETEROLOGOUS EXPRESSION OF PUTATIVE ORs IN *DROSOPHILA MELANOGASTER*

The complete ORF encoding CpomOR3 was amplified by PCR (forward primer 5′-ATGTTTAGTTATGAAAATGAAGACAGC-3′, reverse primer 5′-TCAAGTCATTTCTTCAGTAGAGGT-3′), with antennal cDNA created by the RT-for-PCR kit (Invitrogen) as a template. The purified PCR product was then cloned into the PCR8/GW/TOPO plasmid (Invitrogen). The cassette with the insert was then transferred from the TOPO/GW/PCR8 plasmid to the destination vector (pUASg-HA.attB, constructed by E. Furger and J. Bischof, kindly provided by the Basler group, Zürich), using the Gateway LR Clonase II kit (Invitrogen). The integrity and orientation of the insert was confirmed by sequencing. A transformant *UAS-CpomOR3* line was generated by BestGene (Chino Hills, CA, USA), using the PhiC31 integrase system. Briefly, recombinant pUASg-HA.attB-CpomOR3 plasmids were injected into embryos of a *D. melanogaster* line containing an attP insertion site within the second chromosome (genotype y1 M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP'}ZH-51C), leading to non-random integration. To drive expression of *CpomOR3* in OSNs housed in T1 sensilla, the transformant *UAS-CpomOR3* line was crossed to the $Or67d^{GAL4}$ strain (kindly provided by Barry Dickson) to generate a double homozygous line w^{+};*UAS-CpomOR3*;$Or67d^{GAL4}$. To verify insertion of the *UAS-CpomOR3* construct into the genome, gDNA was extracted and used as template in PCR with primers for the full ORF of *CpomOR3*.

Additionally, to compare the similarity of results between expression sites (trichoid and basiconic sensilla) male flies with the genotype *w*;*UAS-CpomOR3*/CyO;+/+ were mate paired with female flies of the genotype *w;delta-Halo/Cyo;Dmel-UAS-OR22a-Gal4*. This cross drove ectopic expression of *CpomOR3* in the A neuron of the ab3 sensilla, which also expressed the endogenous DmelOR22a receptor in the same neuron. SSR recordings in parental flies from the cross confirmed the absence of any response from DmelOR22a to pear ester (data not shown).

SINGLE SENSILLUM RECORDINGS

The *D. melanogaster* line expressing *CpomOR3* in T1 OSNs, along with the flies expressing *CpomOR3* in ab3A OSNs were tested by SSRs. In all cases, flies were restrained as described in Stensmyr et al. (2003). Briefly, flies were immobilized in 100 µl pipette tips with only the top half of the head protruding. The left

antenna was pushed onto a piece of double-adhesive tape, and held firm by a capillary pressing down from above. Sensilla were contacted with tungsten electrodes (diameter 0.12 mm, Harvard Apparatus Ltd, Edenbridge, United Kingdom) electrolytically sharpened in a saturated KNO_3 solution. A DC-3K micromanipulator equipped with a PM-10 piezo translator (Märzhäuser Wetzler GmbH, Wetzler, Germany) was used to gently maneuver the recording electrode into the base of a sensillum. The reference electrode was inserted through the eye using a DC-3K Rachts PM-10 piezo micromanipulator (Märzhäuser Wetzler GmbH, Wetzler, Germany). The signal from the OSNs was registered and amplified 10 times with a probe (INR-02, Syntech, Hilversum, the Netherlands), and transferred to a computer through an IDAC-4-USB (Syntech) interface, where it was visualized and analyzed with the software Autospike v. 3.4 (Syntech). A constant flow of 0.65 m/s of charcoal-filtered and humidified air was delivered through a glass tube with its outlet approximately 15 mm from the antenna. Stimuli were presented to the insect by inserting a stimulus pipette through a hole in the glass tube, and blowing an air puff of 2.5 ml during 0.5 s through the pipette into the air stream, using a stimulus controller (Syntech SFC-1/b).

Table 1 | Synthetic compounds tested on CpomOR3.

Compound	Biological activity	Source	CAS	Purity (%) (GCMS)
(*E,E*)-8,10-Dodecadienol	Main pheromone component of *C. pomonella*	IRCHA, gift from Prof Heinrich Arn	33956-49-9	98.6 (isomeric purity: 80.1 E,E; 13.6 E,Z; 0.9 Z,E; 5.4 Z,Z)
(*E,Z*)-8,10-Dodecadienol	Synergist for attraction of males of *C. pomonella*	Gift from Prof Rickard Unelius, University of Kalmar, Sweden	33956-50-2	99.8 (isomeric purity: 95.0 E,Z; 0.0 Z,E; 1.5 E,E; 3.5 Z,Z)
(*Z,E*)-8,10-Dodecadienol	Synergist for attraction of males of *C. pomonella*	Gift from Prof Rickard Unelius, University of Kalmar, Sweden	33956-51-3	99.5 (isomeric purity: 84.0 Z,E; 9.9 E,E; 1.7 E,Z; 4.4 Z,Z)
(*Z,Z*)-8,10-Dodecadienol	Antagonist for attraction of males of *C. pomonella*	Gift from Prof Rickard Unelius, University of Kalmar, Sweden	39616-21-2	94.25 (isomeric purity: 77.7 Z,Z; 11.3 Z,E; 2.9 E,E; 8.1 E,Z)
(*E,E*)-8,10-Dodecadienol acetate	Synergist for attraction of males of *C. pomonella*	Bedoukian Inc	53880-51-6	96.2
(*E*)-8-Dodecenol	Minor pheromone component of *C. pomonella*	Voerman, Pherobank	42513-42-8	97
(*E*)-9-Dodecenol	Minor pheromone component of *C. pomonella*	Farchan Labs Inc	35237-62-8	99.7
(*E*)-10-Dodecenol	Minor pheromone component of *C. pomonella*	Voerman, Pherobank	35237-63-9	99.7
1-Dodecanol	Minor pheromone component of *C. pomonella*	Fluka	112-53-8	98.1
(*E*)-β-Farnesene	Synergist for *C. pomonella*	Bedoukian	18794-84-8	98.6
Butyl hexanoate	Synergist for *C. pomonella*	Bedoukian	626-82-4	97.7
Ethyl-(*E,Z*)-2,4-Decadienoate	Synergist for *C. pomonella*	Aldrich	3025-30-7	98.2
(*Z,E*)-9,12-tetradecadienyl acetate	Main pheromone component of *Spodoptera littoralis*	Pherobank	30507-70-1	94.8
4,8-Dimethyl-1, (*E*)-3,7-non-atriene	Antagonist for female attraction of *S. littoralis*	Gift from Prof Wittko Franke, University of Hamburg, Germany	51911-82-1	95
3,7-Dimethyl-1, (*E*)-3,6-octatriene	Antagonist for female attraction of *S. littoralis*	SAFC	3779-61-1	95.4

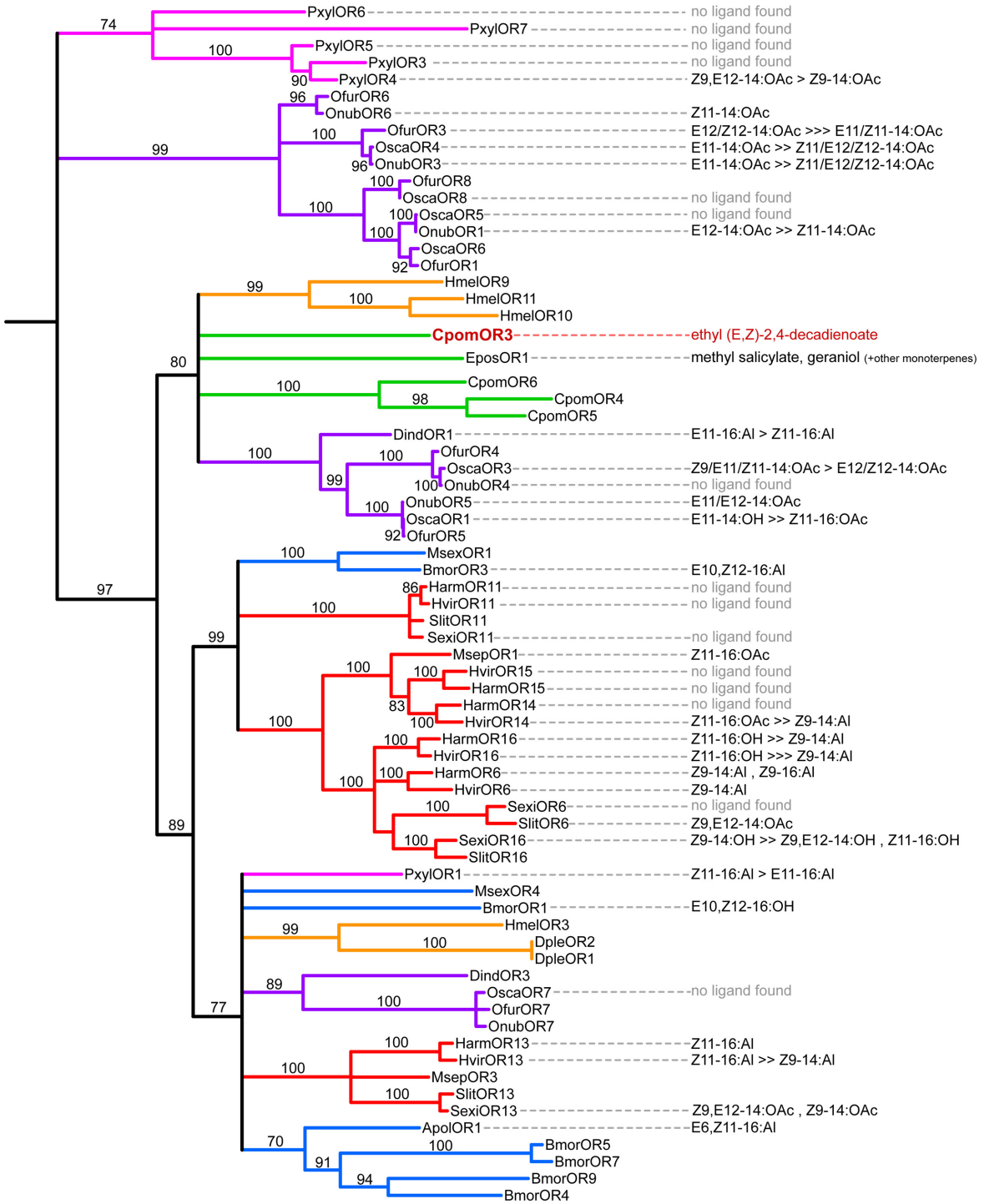

FIGURE 1 | Maximum likelihood tree of lepidopteran candidate PRs, highlighting their corresponding ligand(s). Branch colors represent different lepidopteran lineages: blue for Bombycoidea, red for Noctuoidea, orange for Papilionoidea, purple for Pyraloidea, green for Tortricoidea, and pink for Yponomeutoidea. The outgroup (containing BmorOR6 and HmelOR5, 6, and 7) has been removed from the figure. Functional data has been compiled from the literature (see references in the Methods section). "No ligand found": OR did not respond to any tested pheromone component. Cpom, *Cydia pomonella*, Apol, *Antheraea polyphemus*, Bmor, *Bombyx mori*, Dple, *Danaus plexippus*, Dind, *Diaphania indica*, Epos, *Epiphyas postvittana*, Hmel, *Heliconius melpomene*, Harm, *Helicoverpa armigera*, Hvir, *Heliothis virescens*, Msex, *Manduca sexta*, Msep, *Mythimna separata*, Ofur, *Ostrinia furnacalis*, Onub, *O. nubilalis*, Osca, *O. scapulalis*, Pxyl, *Plutella xylostella*, Sexi, *Spodoptera exigua*, Slit, *S. littoralis*.

SYNTHETIC COMPOUNDS AND ODOR STIMULI

An array of pheromone compounds for *C. pomonella* and related species (Witzgall et al., 1996), as well as known pheromone synergists (El-Sayed, 2014), were tested on CpomOR3 (**Table 1**). Combinations of the *C. pomonella* main pheromone compound, codlemone, with the synergists were also tested, as they have previously been shown to create distinct activation patterns in the antennal lobe, the primary olfactory center, compared to either compound alone (Trona et al., 2013). Purity of compounds was estimated by GC-MS.

Stimuli were prepared by applying compounds to 1.5 × 1 cm pieces of filter paper that were placed in disposable glass Pasteur pipettes (VWR International, Stockholm, Sweden). Truncated 1 ml pipette tips were put on the wide end of the Pasteur pipettes, to reduce evaporation of the test compound(s). Compounds were diluted in hexane (redistilled from 95%, Labscan, Dublin, Ireland). A volume of 10 µl of a 1 µg/µl solution was applied to filter papers for a total amount of 10 µg per stimulus. The same dilution procedure was used in dose-response experiments, except that compounds were diluted to concentrations ranging from 0.1 ng/µl to 10 µg/µl in decadic steps, to achieve different concentrations when 10 µl of the diluted compound were applied to the filter paper in the stimulus pipette. Control stimuli with only solvent were also prepared. Fresh stimuli were prepared before each recording session, and kept at −18°C until the start of the recording session, to avoid evaporation. Only complete recording sessions of the entire set of test stimuli were evaluated, and only one screening or dose response session was performed from a single sensillum per individual. A total of 16 screenings were performed, while for dose response experiments, 10 replicates were performed.

Responses were quantified by counting the number of spikes for 500 ms starting from the onset of response (as determined by the earliest response for the recording session), subtracting the number of spikes during the 500 ms before response, and doubling this value to get the response in Hz (spikes/s). Responses of T1 sensilla to different pheromone and pheromone synergist compounds were compared using ANOVA with repeated measures, while responses to different doses of pear ester with the two types of sensilla evaluated were compared with Two-Way ANOVA. All statistical analyses were performed using SPSS Version 19.0 (IBM Corp., Armonk, NY, USA).

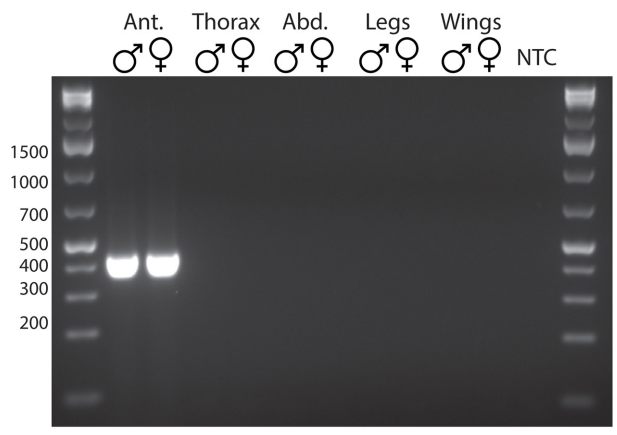

FIGURE 2 | Reverse transcription PCR showing antennal specific expression of *C. pomonella* OR3 in both sexes. Ant., antennae, Abd., abdomen, NTC, no template control.

RESULTS

CLONING OF THE OPEN READING FRAME OF *CPOMOR3* AND SEQUENCE ANALYSIS

The partial *CpomOR3* sequence (Bengtsson et al., 2012), judged to be complete at the 3′ end based on the presence of a stop codon, but not at the 5′ end, was extended by 5′ RACE-PCR. Merging the sequence of the 1096 bp 5′RACE-PCR product we obtained together with the previous sequence led to a 1281 bp transcript, containing the complete ORF of *CpomOR3*, confirmed by alignment of the deduced protein with other lepidopteran ORs. The full ORF sequence for *CpomOR3* was further amplified and sequenced to verify the absence of chimera. The full sequence has been submitted to Genbank (accession number KJ420588). The TMHMM2.0 model predicted 6 transmembrane domains for CpomOR3. CpomOR3 exhibits a mean sequence identity of 34.3% with other PRs, with a maximum identity of 41.4% with *Diaphania indica* OR1. Alignment with lepidopteran candidate PRs did not reveal any notable feature of CpomOR3, apart from a serine residue—also present in other tortricid sequences—located within the final transmembrane domain (position 296), instead of the glycine residue found in all the other lepidopteran PR sequences.

PHYLOGENY OF LEPIDOPTERAN CANDIDATE PRs

A maximum likelihood phylogeny was built from a large dataset containing CpomOR3 to 6—the putative *C. pomonella* PRs (Bengtsson et al., 2012)—and 70 other candidate PR full-length sequences. In this tree (**Figure 1**), the candidate PRs grouped within five large sub-clades within the PR clade. All the sequences from tortricid moths (*C. pomonella* and *E. postvittana*, green branches), including CpomOR3, clustered within one of these five clades (supported by a bootstrap value of 80), albeit the exact relationships between CpomOR3 and the other receptors of this clade were not resolved due to low bootstrap support values (to reflect lack of support, nodes with a bootstrap value lower than 70 were collapsed). Even if the CpomOR1 sequence was not part of this dataset because of its short length, it also clustered in the same clade during previous analyses, as a sister group to EposOR1 (data not shown). All the PR candidates from *C. pomonella* characterized to date thus have a relatively recent common origin, in spite of their low sequence identity levels.

TISSUE-RELATED EXPRESSION OF CpomOR3

Reverse transcription PCR showed a clear expression pattern for *CpomOR3*, with strong expression in antennae, but not in other body parts (**Figure 2**). Moreover, there was no sex-specific expression of *CpomOR3*, as it appeared to be expressed in antennae of both males and females.

RESPONSE SPECTRUM OF CpomOR3 TO PUTATIVE LIGANDS

Single-sensillum recordings from transformed *Drosophila* line expressing CpomOR3 in T1 OSNs revealed that these neurons only responded to pear ester (41 spikes/s, $N = 16$) out of 15 compounds. Six different mixtures of different combinations of pheromone components and plant compounds were also tested, and only the one that contained pear ester and codlemone elicited a significant response (**Figure 3**). No synergy between these two compounds was observed (Bonferroni *post-hoc* test). Dose response experiments established the threshold of response to pear ester to be at 10 µg for both trichoid T1 and ab3A OSNs (**Figure 4**).

DISCUSSION

CpomOR3 IS TUNED TO THE PLANT VOLATILE PEAR ESTER

Electrophysiological recordings from *Drosophila* basiconic ab3 and trichoid T1 sensilla, housing OSNs heterologously expressing CpomOR3, demonstrate that CpomOR3 is tuned to pear

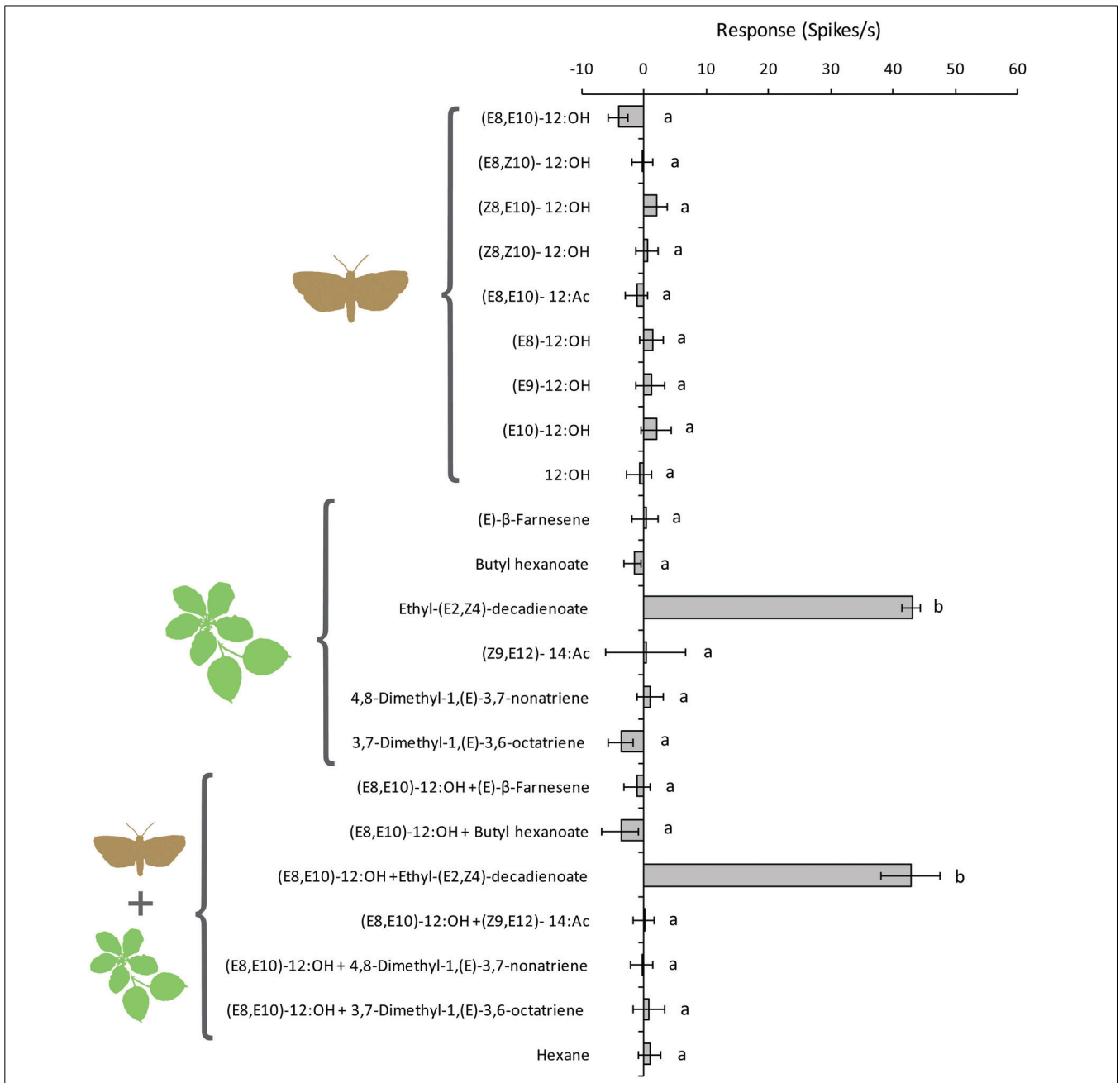

FIGURE 3 | Response (in Hz) of *D. melanogaster* T1 OSNs expressing CpomOR3 to stimulation with known *C. pomonella* pheromone components, plant-related synergists, and combinations of the main pheromone component codlemone, with plant synergists. Letters denote subgroups with a statistically significant separation (Repeated measures ANOVA with Bonferroni *post-hoc* test, $n = 16$).

ester, ethyl (*E,Z*)-2,4-decadienoate (**Figures 3**, **4**). Reverse transcription PCR suggests that CpomOR3 is expressed without sex bias in the antennae of both males and females (**Figure 2**). This finding matches the behavioral evidence, since pear ester is a bisexual codling moth attractant (Light et al., 2001; Light and Knight, 2005). The existence of a dedicated receptor corroborates the significance of pear ester for host plant detection in codling moth males and females, and contributes to current research aiming at a complete identification of codling moth host plant attractants.

Results from these heterologous expression studies confirm previous recordings obtained from codling moth antennae, showing presence of OSNs responding to pear ester (De Cristofaro et al., 2004; Ansebo et al., 2005). However, a spatially tight arrangement of sensilla on codling moth antennae renders it difficult to obtain replicated recordings from the same sensillum type, and to differentiate between responses from co-localized OSNs in the same sensillum, or even from OSNs in adjacent sensilla (Lee and Baker, 2008). This further demonstrates the appreciable addition of heterologous OR expression in *Drosophila* to the toolbox for identification of behaviorally relevant plant odorants.

Intracellular recordings of axons of OSNs projecting to the antennal lobe (AL), the olfactory center of the insect brain, and functional imaging of AL glomeruli, receiving input from OSNs expressing the same ORs, support our finding that pear ester activates a dedicated olfactory channel and that interaction of pear ester with other compounds, including the sex pheromone codlemone, takes place in the AL, and not at the periphery (**Figure 3**; Trona et al., 2010, 2013).

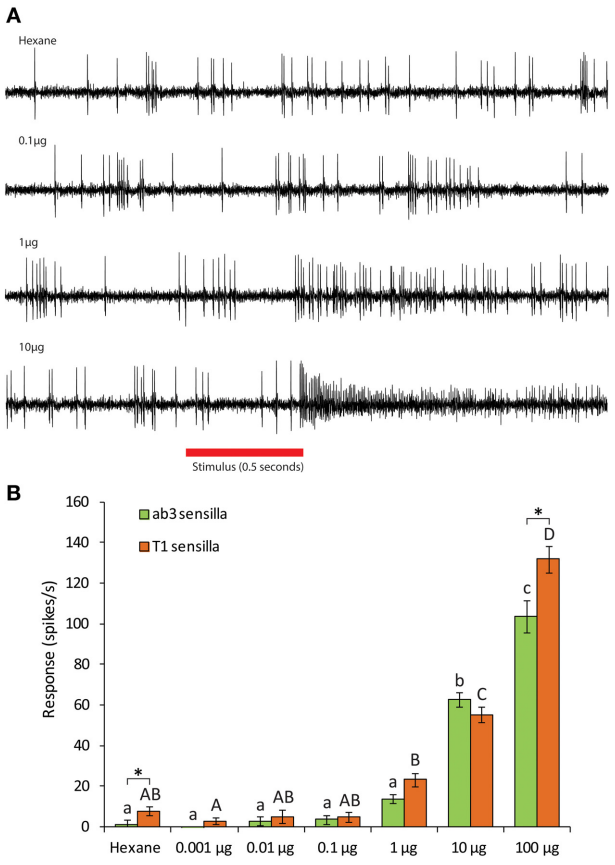

FIGURE 4 | (A) Traces of single sensillum recordings from *D. melanogaster* T1 sensilla expressing CpomOR3 to pear ester at different doses. **(B)** Dose-dependent response of CpomOR3 to pear ester in different types of sensilla. Bars of the same color followed by different letters indicate subgroups with statistically significant differences. Asterisks denote significant differences among different types of sensilla for the dose indicated (Two-Way ANOVA with Tukey's *post-hoc* test, $p < 0.05$, $n = 10$).

CpomOR3 BELONGS TO THE PHEROMONE RECEPTOR CLADE

CpomOR3 belongs to the conserved clade of lepidopteran pheromone receptors (**Figure 1**), although it binds to pear ester only and to none of the pheromonal compounds produced by *C. pomonella* females or closely related *Cydia* species (Witzgall et al., 1996, 2001). CpomOR3 was almost equally sensitive when expressed in trichoid T1 and basiconic ab3 sensilla, except at the highest dose of pear ester (**Figure 4**). Interestingly, the pheromone receptors BmorOR1 of silkmoth *B. mori* and SlitOR6 of cotton leafworm moth *S. littoralis* were more sensitive when expressed in T1 than in ab3 sensilla (Syed et al., 2010; Montagné et al., 2012). This indicates that T1 sensilla, containing an important PR partner, the sensory neuron membrane protein (Benton et al., 2007), are more adapted for correct PR functioning, whereas plant odorant ORs function equally well in T1 or ab3.

In addition, the demonstration that an OR clustering in the PR clade is a plant odorant receptor offers an explanation for the lack of a response of orphan lepidopteran PRs to pheromone compounds (Wang et al., 2010; Liu et al., 2013). Phylogenetic analysis confirms that the lepidopteran PR clade contains another co-evolved receptor for plant compounds, EposOR1, from another tortricid species, the light brown apple moth *E. postvittana*. The strongest ligand for EposOR1 is a common plant compound, methyl salicylate (Jordan et al., 2009), which has a behavioral effect in many insects (**Figure 1**; El-Sayed, 2014). With the currently available sequence and functional data, phylogenetic analysis cannot resolve if EposOR1 and CpomOR3 have a single ancestor, or if two unique evolutionary events gave rise to these plant volatile receptors within the PR clade (**Figure 1**). However, both CpomOR3 and EposOR1 belong to the same clade, which notably also contains the four other *C. pomonella* candidate PRs (Bengtsson et al., 2012; Garczynski et al., 2012). Further studies, using both pheromones and plant volatiles, will help to understand the functional divergence of the PR clade.

INTERACTION BETWEEN PEAR ESTER AND CODLING MOTH PHEROMONE

The finding that a codling moth PR is tuned to pear ester is remarkable. It corroborates the interaction between pear ester and codlemone, which may play an important role in codling moth premating communication and reproductive isolation (Trona et al., 2013).

Axons of OSNs expressing the same OR or PR genes converge onto the same glomerulus in the antennal lobe (AL). Since each OR corresponds to a glomerulus in the AL, it follows that new

glomeruli arise during OR repertoire expansion. Indeed, closely related ORs with high sequence similarity are often expressed in OSNs that project to neighboring glomeruli in the AL (Couto et al., 2005; Masse et al., 2009; Ramdya and Benton, 2010; Cande et al., 2013).

Accordingly, the architecture of the codling moth AL lends support to the hypothesis that the OR genes for pear ester and codlemone, the codling moth sex pheromone, are closely related—the glomeruli dedicated to pear ester and codlemone are adjacent glomeruli in the codling moth AL, where stimulation with a blend of codlemone and pear ester produces a very strong synergistic effect (Trona et al., 2010, 2013). Although the PR for codlemone has not yet been found, we can reasonably assume that it belongs to the PR clade, which contains the putative pheromone receptors CpomOR1, and CpomOR4 through 6 (**Figure 1**; Bengtsson et al., 2012).

Chemosensory receptor genes arise by gene duplication and progressively diverge following adaptive changes. In *Drosophila*, phylogenetically related chemosensory genes on a chromosome tend to be located closely together on a chromosome (Nei et al., 2008; Sanchez-Gracia et al., 2009). Physically neighboring chemosensory genes restrict genetic recombination and thus become a combined target for selection. Tight physical linkage between host performance and preference genes, leading to assortative mating through habitat choice, has been first discovered in pea aphids (Hawthorne and Via, 2001; Smadja et al., 2012). Key traits that are associated via linkage and which combine ecological and sexual selection are particularly powerful during phylogenetic divergence (Servedio et al., 2011; Merrill et al., 2012; Safran et al., 2013).

In codling moth, chemosensory receptor genes encoding host preference and mate recognition, tuned to the plant volatile pear ester and sex pheromone, are expected to be associated to facilitate host adaptation and reproductive isolation in concert. This hypothesis can be tested after the receptor gene for codlemone has been found.

REFERENCES

Abascal, F., Zardoya, R., and Posada, D. (2005). ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* 21, 2104–2105. doi: 10.1093/bioinformatics/bti263

Ansebo, L., Ignell, R., Löfqvist, J., and Hansson, B. S. (2005). Responses to sex pheromone and plant odours by olfactory receptor neurons housed in sensilla auricillica of the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). *J. Insect Physiol.* 51, 1066–1074. doi: 10.1016/j.jinsphys.2005.05.003

Bengtsson, J. M., Trona, F., Montagné, N., Anfora, G., Ignell, R., Witzgall, P., et al. (2012). Putative chemosensory receptors of the codling moth, *Cydia pomonella*, identified by antennal transcriptome analysis. *PLoS ONE* 7:e31620. doi: 10.1371/journal.pone.0031620

Bengtsson, M., Bäckman, A.-C., Liblikas, I., Ramirez, M. I., Borg-Karlson, A.-K., Ansebo, L., et al. (2001). Plant odor analysis of apple: antennal response of codling moth females to apple volatiles during phenological development. *J. Agric. Food Chem.* 49, 3736–3741. doi: 10.1021/jf0100548

Bengtsson, M., Jaastad, G., Knudsen, G., Kobro, S., Bäckman, A.-C., Pettersson, E., et al. (2006). Plant volatiles mediate attraction to host and non-host plant in apple fruit moth, *Argyresthia conjugella*. *Entomol. Exp. Appl.* 118, 77–85. doi: 10.1111/j.1570-7458.2006.00359.x

Benton, R., Vannice, K. S., and Vosshall, L. B. (2007). An essential role for a CD36-related receptor in pheromone detection in *Drosophila*. *Nature* 450, 289–293. doi: 10.1038/nature06328

Berger, R. G., and Drawert, F. (1984). Changes in the composition of volatiles by post-harvest application of alcohols to red delicious apples. *J. Sci. Food Agric.* 35, 1318–1325. doi: 10.1002/jsfa.2740351208

Beroza, M., Bierl, B. A., and Moffitt, H. R. (1974). Sex pheromones: (E,E)-8,10-dodecadien-1-ol in the codling moth. *Science* 183, 89–90. doi: 10.1126/science.183.4120.89

Bruce, T. J. A., and Pickett, J. A. (2011). Perception of plant volatile blends by herbivorous insects—finding the right mix. *Phytochemistry* 72, 1605–1611. doi: 10.1016/j.phytochem.2011.04.011

Cande, J., Prud'homme, B., and Gompel, N. (2013). Smells like evolution: the role of chemoreceptor evolution in behavioral change. *Curr. Opin. Neurobiol.* 23, 152–158. doi: 10.1016/j.conb.2012.07.008

Cha, D. H., Linn, C. E., Teal, P. E. A., Zhang, A. J., Roelofs, W. L., and Loeb, G. M. (2011). Eavesdropping on plant volatiles by a specialist moth: significance of ratio and concentration. *PLoS ONE* 6:e17033. doi: 10.1371/journal.pone.0017033

Coracini, M., Bengtsson, M., Liblikas, I., and Witzgall, P. (2004). Attraction of codling moth males to apple volatiles. *Entomol. Exp. Appl.* 110, 1–10. doi: 10.1111/j.0013-8703.2004.00124.x

Couto, A., Alenius, M., and Dickson, B. J. (2005). Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Curr. Biol.* 15, 1535–1547. doi: 10.1016/j.cub.2005.07.034

De Cristofaro, A., Ioriatti, C., Pasqualini, E., Anfora, G., Germinara, G. S., Villa, M., et al. (2004). Electrophysiological responses of *Cydia pomonella* (L.) to codlemone and pear ester ethyl (E,Z)-2,4-decadienoate: peripheral interactions in their perception and evidences for cells responding to both compounds. *Bull. Insectol.* 57, 137–144.

Dethier, V. G. (1947). *Chemical Insect Attractants and Repellents*. Philadelphia, PA: Blakiston.

Dethier, V. G. (1982). Mechanism of host plant recognition. *Entomol. Exp. Appl.* 31, 49–56. doi: 10.1111/j.1570-7458.1982.tb03118.x

Dobritsa, A. A., van der Goes van Naters, W., Warr, C. G., Steinbrecht, R. A., and Carlson, J. R. (2003). Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron* 37, 827–841. doi: 10.1016/S0896-6273(03)00094-1

Dres, M., and Mallet, J. (2002). Host races in plant-feeding insects and their importance in sympatric speciation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 357, 471–492. doi: 10.1098/rstb.2002.1059

Ehrlich, P., and Raven, P. (1964). Butterflies and plants: a study in coevolution. *Evolution* 18, 586–608. doi: 10.2307/2406212

El-Sayed, A. M. (2014). *The Pherobase: Database of Pheromones and Semiochemicals.* Available online at: www.pherobase.com

Forstner, M., Breer, H., and Krieger, J. (2009). A receptor and binding protein interplay in the detection of a distinct pheromone component in the silkmoth *Antheraea polyphemus*. *Int. J. Biol. Sci.* 5:745. doi: 10.7150/ijbs.5.745

Garczynski, S. F., Wanner, K. W., and Unruh, T. R. (2012). Identification and initial characterization of the 3′ end of gene transcripts encoding putative members of the pheromone receptor subfamily in Lepidoptera. *Insect Sci.* 19, 64–74. doi: 10.1111/j.1744-7917.2011.01423.x

Grosse-Wilde, E., Gohl, T., Bouche, E., Breer, H., and Krieger, J. (2007). Candidate pheromone receptors provide the basis for the response of distinct antennal neurons to pheromonal compounds. *Eur. J. Neurosci.* 25, 2364–2373. doi: 10.1111/j.1460-9568.2007.05512.x

Grosse-Wilde, E., Stieber, R., Forstner, M., Krieger, J., Wicher, D., and Hansson, B. S. (2010). Sex-specific odorant receptors of the tobacco hornworm *Manduca sexta*. *Front. Cell. Neurosci.* 4:22. doi: 10.3389/fncel.2010.00022

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* 59, 307–321. doi: 10.1093/sysbio/syq010

Hallem, E. A., and Carlson, J. R. (2006). Coding of odors by a receptor repertoire. *Cell* 125, 143–160. doi: 10.1016/j.cell.2006.01.050

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. doi: 10.1016/j.cell.2004.05.012

Hawthorne, D. J., and Via, S. (2001). Genetic linkage of ecological specialization and reproductive isolation in pea aphids. *Nature* 412, 904–907. doi: 10.1038/35091062

Heliconius Genome Consortium. (2012). Butterfly genome reveals promiscuous exchange of mimicry adaptations among species. *Nature* 487, 94–98. doi: 10.1038/nature11041

Hern, A., and Dorn, S. (2004). A female-specific attractant for the codling moth, *Cydia pomonella*, from apple fruit volatiles. *Naturwissenschaften* 91, 77–80. doi: 10.1007/s00114-003-0484-6

Ihara, S., Yoshikawa, K., and Touhara, K. (2013). Chemosensory signals and their receptors in the olfactory neural system. *Neuroscience* 254, 45–60. doi: 10.1016/j.neuroscience.2013.08.063

Jacquin-Joly, E., and Merlin, C. (2004). Insect olfactory receptors: contributions of molecular biology to chemical ecology. *J. Chem. Ecol.* 30, 2359–2397. doi: 10.1007/s10886-004-7941-3

Jennings, W. G., Heinz, D. E., and Creveling, R. K. (1964). Volatile esters of Bartlett pear. IV. Esters of trans-2-cis-4-decadienoic acid. *J. Food Sci.* 29, 730–734. doi: 10.1111/j.1365-2621.1964.tb00439.x

Jiang, X.-J., Guo, H., Di, C., Yu, S., Zhu, L., Huang, L.-Q., et al. (2014). Sequence similarity and functional comparisons of pheromone receptor orthologs in two closely related *Helicoverpa* species. *Insect Biochem. Mol. Biol.* 48, 63–74. doi: 10.1016/j.ibmb.2014.02.010

Jones, W. D., Nguyen, T. A. T., Kloss, B., Lee, K. J., and Vosshall, L. B. (2005). Functional conservation of an insect odorant receptor gene across 250 million years of evolution. *Curr. Biol.* 15, R119–R121. doi: 10.1016/j.cub.2005.02.007

Jordan, M. D., Anderson, A., Begum, D., Carraher, C., Authier, A., Marshall, S. D. G., et al. (2009). Odorant receptors from the light brown apple moth (*Epiphyas postvittana*) recognize important volatile compounds produced by plants. *Chem. Senses* 34, 383–394. doi: 10.1093/chemse/bjp010

Katoh, K., Kuma, K.-I., Toh, H., and Miyata, T. (2005). MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Res.* 33, 511–518. doi: 10.1093/nar/gki198

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Knight, A., Light, D., and Chebny, V. (2013). Monitoring codling moth (Lepidoptera: Tortricidae) in orchards treated with pear ester and sex pheromone combo dispensers. *J. Appl. Entomol.* 137, 214–224. doi: 10.1111/j.1439-0418.2012.01715.x

Knight, A. L., and Light, D. M. (2001). Attractants from Bartlett pear for codling moth, *Cydia pomonella* (L.), larvae. *Naturwissenschaften* 88, 339–342. doi: 10.1007/s001140100244

Knight, A. L., and Light, D. M. (2012). Monitoring codling moth (Lepidoptera: Tortricidae) in sex pheromone-treated orchards with (E)-4,8-dimethyl-1,3,7-nonatriene or pear ester in combination with codlemone and acetic acid. *Environ. Entomol.* 41, 407–414. doi: 10.1603/EN11310

Knight, A. L., and Light, D. M. (2013). Adding microencapsulated pear ester to insecticides for control of *Cydia pomonella* (Lepidoptera: Tortricidae) in apple. *Pest Manag. Sci.* 69, 66–74. doi: 10.1002/ps.3363

Knight, A. L., Stelinski, L. L., Hebert, V., Gut, L., Light, D., and Brunner, J. (2012). Evaluation of novel semiochemical dispensers simultaneously releasing pear ester and sex pheromone for mating disruption of codling moth (Lepidoptera: Tortricidae). *J. Appl. Entomol.* 136, 79–86. doi: 10.1111/j.1439-0418.2011.01633.x

Kreher, S. A., Kwon, J. Y., and Carlson, J. R. (2005). The molecular basis of odor coding in the *Drosophila* larva. *Neuron* 46, 445–456. doi: 10.1016/j.neuron.2005.04.007

Krieger, J., Klink, O., Mohl, C., Raming, K., and Breer, H. (2003). A candidate olfactory receptor subtype highly conserved across different insect orders. *J. Comp. Physiol. A* 189, 519–526. doi: 10.1007/s00359-003-0427-x

Kurtovic, A., Widmer, A., and Dickson, B. J. (2007). A single class of olfactory neurons mediates behavioural responses to a *Drosophila* sex pheromone. *Nature* 446, 542–546. doi: 10.1038/nature05672

Le, S. Q., and Gascuel, O. (2008). An improved general amino acid replacement matrix. *Mol. Biol. Evol.* 25, 1307–1320. doi: 10.1093/molbev/msn067

Leal, W. S. (2013). Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. *Annu. Rev. Entomol.* 58, 373–391. doi: 10.1146/annurev-ento-120811-153635

Leary, G. P., Allen, J. E., Bunger, P. L., Luginbill, J. B., Linn, C. E., Macallister, I. E., et al. (2012). Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14081–14086. doi: 10.1073/pnas.1204661109

Lee, S. G., and Baker, T. C. (2008). Incomplete electrical isolation of sex-pheromone responsive olfactory receptor neurons from neighboring sensilla. *J. Insect Physiol.* 54, 663–671. doi: 10.1016/j.jinsphys.2008.01.006

Legeai, F., Malpel, S., Montagné, N., Monsempes, C., Cousserans, F., Merlin, C., et al. (2011). An expressed sequence tag collection from the male antennae of the Noctuid moth *Spodoptera littoralis*: a resource for olfactory and pheromone detection research. *BMC Genomics* 12:86. doi: 10.1186/1471-2164-12-86

Letunic, I., and Bork, P. (2011). Interactive tree of life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res.* 39, W475–W478. doi: 10.1093/nar/gkr201

Light, D. M., and Beck, J. J. (2012). Behavior of codling moth (Lepidoptera: Tortricidae) neonate larvae on surfaces treated with microencapsulated pear ester. *Environ. Entomol.* 41, 603–611. doi: 10.1603/EN11273

Light, D. M., and Knight, A. (2005). Specificity of codling moth (Lepidoptera: Tortricidae) for the host plant kairomone, ethyl (2E,4Z)-2,4-decadienoate: field bioassays with pome fruit volatiles, analogue, and isomeric compounds. *J. Agric. Food Chem.* 53, 4046–4053. doi: 10.1021/jf040431r

Light, D. M., Knight, A. L., Henrick, C. A., Rajapaska, D., Lingren, B., Dickens, J. C., et al. (2001). A pear-derived kairomone with pheromonal potency that attracts male and female codling moth, *Cydia pomonella* (L.). *Naturwissenschaften* 88, 333–338. doi: 10.1007/s001140100243

Liu, C., Liu, Y., Walker, W. B., Dong, S., and Wang, G. (2013). Identification and functional characterization of sex pheromone receptors in beet armyworm *Spodoptera exigua* (Hübner). *Insect Biochem. Mol. Biol.* 43, 747–754. doi: 10.1016/j.ibmb.2013.05.009

Liu, Y., Gu, S., Zhang, Y., Guo, Y., and Wang, G. (2012). Candidate olfaction genes identified within the *Helicoverpa armigera* antennal transcriptome. *PLoS ONE* 7:e48260. doi: 10.1371/journal.pone.0048260

Masse, N. Y., Turner, G. C., and Jefferis, G. S. X. E. (2009). Olfactory information processing in *Drosophila*. *Curr. Biol.* 19, R700–R713. doi: 10.1016/j.cub.2009.06.026

Matsubayashi, K. W., Ohshima, I., and Nosil, P. (2010). Ecological speciation in phytophagous insects. *Entomol. Exp. Appl.* 134, 1–27. doi: 10.1111/j.1570-7458.2009.00916.x

Merrill, R. M., Wallbank, R. W. R., Bull, V., Salazar, P. C. A., Mallet, J., Stevens, M., et al. (2012). Disruptive ecological selection on a mating cue. *Proc. Biol. Sci.* 279, 4907–4913. doi: 10.1098/rspb.2012.1968

Mitsuno, H., Sakurai, T., Murai, M., Yasuda, T., Kugimiya, S., Ozawa, R., et al. (2008). Identification of receptors of main sex pheromone components of three Lepidopteran species. *Eur. J. Neurosci.* 28, 893–902. doi: 10.1111/j.1460-9568.2008.06429.x

Miura, N., Nakagawa, T., Tatsuki, S., Touhara, K., and Ishikawa, Y. (2009). A male-specific odorant receptor conserved through the evolution of sex pheromones in *Ostrinia* moth species. *Int. J. Biol. Sci.* 5:319. doi: 10.7150/ijbs.5.319

Miura, N., Nakagawa, T., Touhara, K., and Ishikawa, Y. (2010). Broadly and narrowly tuned odorant receptors are involved in female sex pheromone reception in *Ostrinia* moths. *Insect Biochem. Mol. Biol.* 40, 64–73. doi: 10.1016/j.ibmb.2009.12.011

Montagné, N., Chertemps, T., Brigaud, I., Francois, A., Francois, M. C., de Fouchier, A., et al. (2012). Functional characterization of a sex pheromone receptor in the pest moth *Spodoptera littoralis* by heterologous expression in *Drosophila*. *Eur. J. Neurosci.* 36, 2588–2596. doi: 10.1111/j.1460-9568.2012.08183.x

Nakagawa, T., Sakurai, T., Nishioka, T., and Touhara, K. (2005). Insect sex-pheromone signals mediated by specific combinations of olfactory receptors. *Science* 307, 1638–1642. doi: 10.1126/science.1106267

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. doi: 10.1038/nrg2480

Pinero, J. C., Galizia, C. G., and Dorn, S. (2008). Synergistic behavioural responses of female oriental fruit moths (Lepidoptera: Tortricidae) to syntetic host plant-derived mixtures are mirrored by odor-evoked calcium activity in their antennal lobes. *J. Insect Physiol.* 54, 333–343. doi: 10.1016/j.jinsphys.2007.10.002

Poivet, E., Gallot, A., Montagné, N., Glaser, N., Legeai, F., and Jacquin-Joly, E. (2013). A comparison of the olfactory gene repertoires of adults and larvae in the noctuid moth *Spodoptera littoralis*. *PLoS ONE* 8:e60263. doi: 10.1371/journal.pone.0060263

Ramdya, P., and Benton, R. (2010). Evolving olfactory systems on the fly. *Trends Genet.* 26, 307–316. doi: 10.1016/j.tig.2010.04.004

Riffell, J. A., Lei, H., and Hildebrand, J. G. (2009). Neural correlates of behavior in the moth *Manduca sexta* in response to complex odors. *Proc. Natl. Acad. Sci. U.S.A.* 106, 19219–19226. doi: 10.1073/pnas.0910592106

Roelofs, W. L., Comeau, A., Hill, A., and Milicevic, G. (1971). Sex attractant of the codling moth: characterization with electroantennogram technique. *Science* 174, 297–299. doi: 10.1126/science.174.4006.297

Safran, R. J., Scordato, E. S. C., Symes, L. B., Rodriguez, R. L., and Mendelson, T. C. (2013). Contributions of natural and sexual selection to the evolution of premating reproductive isolation: a research agenda. *Trends Ecol. Evol.* 28, 643–650. doi: 10.1016/j.tree.2013.08.004

Sakurai, T., Nakagawa, T., Mitsuno, H., Mori, H., Endo, Y., Tanoue, S., et al. (2004). Identification and functional characterization of a sex pheromone receptor in the silkmoth *Bombyx mori*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16653–16658. doi: 10.1073/pnas.0407596101

Sanchez-Gracia, A., Vieira, F. G., and Rozas, J. (2009). Molecular evolution of the major chemosensory gene families in insects. *Heredity* 103, 208–216. doi: 10.1038/hdy.2009.55

Schmidt-Busser, D., von Arx, M., Connetable, S., and Guerin, P. M. (2011). Identification of host-plant chemical stimuli for the European grape berry moth *Eupoecilia ambiguella*. *Physiol. Entomol.* 36, 101–110. doi: 10.1111/j.1365-3032.2010.00766.x

Servedio, M. R., Van Doorn, G. S., Kopp, M., Frame, A. M., and Nosil, P. (2011). Magic traits in speciation: "magic" but not rare? *Trends Ecol. Evol.* 26, 389–397. doi: 10.1016/j.tree.2011.04.005

Smadja, C., and Butlin, R. K. (2009). On the scent of speciation: the chemosensory system and its role in premating isolation. *Heredity* 102, 77–97. doi: 10.1038/hdy.2008.55

Smadja, C. M., Canback, B., Vitalis, R., Gautier, M., Ferrari, J., Zhou, J. J., et al. (2012). Large-scale candidate gene scan reveals the role of chemoreceptor genes in host plant specialization an speciation in the pea aphid. *Evolution* 66, 2723–2738. doi: 10.1111/j.1558-5646.2012.01612.x

Stensmyr, M. C., Dekker, T., and Hansson, B. S. (2003). Evolution of the olfactory code in the *Drosophila melanogaster* subgroup. *Proc. Biol. Sci.* 270, 2333–2340. doi: 10.1098/rspb.2003.2512

Sun, M., Liu, Y., Walker, W. B., Liu, C., Lin, K., Gu, S., et al. (2013). Identification and characterization of pheromone receptors and interplay between receptors and pheromone binding proteins in the diamondback moth, *Plutella xyllostella*. *PLoS ONE* 8:e62098. doi: 10.1371/journal.pone.0062098

Syed, Z., Kopp, A., Kimbrell, D. A., and Leal, W. S. (2010). Bombykol receptors in the silkworm moth and the fruit fly. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9436–9439. doi: 10.1073/pnas.1003881107

Tasin, M., Bäckman, A.-C., Anfora, G., Carlin, S., Ioriatti, C., and Witzgall, P. (2010). Attraction of female grapevine moth to common and specific olfactory cues from 2 host plants. *Chem. Senses* 35, 57–64. doi: 10.1093/chemse/bjp082

Tasin, M., Bäckman, A.-C., Bengtsson, M., Varela, N., Ioriatti, C., and Witzgall, P. (2006). Wind tunnel attraction of grapevine moth females, *Lobesia botrana*, to natural and artificial grape odour. *Chemoecology* 16, 87–92. doi: 10.1007/s00049-005-0332-6

Thoming, G., and Knudsen, G. K. (2014). Attraction of pea moth *Cydia nigricana* to pea flower volatiles. *Phytochemistry* 100, 66–75. doi: 10.1016/j.phytochem.2014.01.005

Trona, F., Anfora, G., Balkenius, A., Bengtsson, M., Tasin, M., Knight, A., et al. (2013). Neural coding merges sex and habitat chemosensory signals in an insect herbivore. *Proc. Biol. Sci.* 280:20130267. doi: 10.1098/rspb.2013.0267

Trona, F., Anfora, G., Bengtsson, M., Witzgall, P., and Ignell, R. (2010). Coding and interaction of sex pheromone and plant volatile signals in the antennal lobe of the codling moth *Cydia pomonella*. *J. Exp. Biol.* 213, 4291–4303. doi: 10.1242/jeb.047365

Wang, G., Vásquez, G., Schal, C., Zwiebel, L., and Gould, F. (2010). Functional characterization of pheromone receptors in the tobacco budworm *Heliothis virescens*. *Insect Mol. Biol.* 20, 125–133. doi: 10.1111/j.1365-2583.2010.01045.x

Wanner, K. W., Nichols, A. S., Allen, J. E., Bunger, P. L., Garczynski, S. F., Linn, C. E., et al. (2010). Sex pheromone receptor specificity in the European corn borer moth, *Ostrinia nubilalis*. *PLoS ONE* 5:e8685. doi: 10.1371/journal.pone.0008685

Willner, B., Granvogl, M., and Schieberle, P. (2013). Characterization of the key aroma compounds in Bartlett pear brandies by means of the sensomics concept. *J. Agric. Food Chem.* 61, 9583–9593. doi: 10.1021/jf403024t

Witzgall, P., Ansebo, L., Yang, Z., Angeli, G., Sauphanor, B., and Bengtsson, M. (2005). Plant volatiles affect oviposition by codling moths. *Chemoecology* 15, 77–83. doi: 10.1007/s00049-005-0295-7

Witzgall, P., Bengtsson, M., Rauscher, S., Liblikas, I., Bäckman, A.-C., Coracini, M., et al. (2001). Identification of further sex pheromone synergists in the codling moth, *Cydia pomonella*. *Entomol. Exp. Appl.* 101, 131–141. doi: 10.1046/j.1570-7458.2001.00898.x

Witzgall, P., Chambon, J.-P., Bengtsson, M., Unelius, C. R., Appelgren, M., Makranczy, G., et al. (1996). Sex pheromones and attractants in the Eucosmini and Grapholitini (Lepidoptera, Tortricidae). *Chemoecology* 7, 13–23. doi: 10.1007/BF01240633

Zhan, S., Merlin, C., Boore, J. L., and Reppert, S. M. (2011). The monarch butterfly genome yields insights into long-distance migration. *Cell* 147, 1171–1185. doi: 10.1016/j.cell.2011.09.052

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Alternative splicing produces two transcripts encoding female-biased pheromone subfamily receptors in the navel orangeworm, *Amyelois transitella*

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Insect odorant receptors (ORs) are key sensors of environmental odors and members of the lepidopteran pheromone receptor subfamily are thought to play important roles in mate finding by recognizing sex pheromones. Much research has been done to identify putative pheromone receptors in lepidopteran males, but little attention has been given to female counterparts. In this study, degenerate oligonucleotide primers designed against a conserved amino acid region in the C-terminus of lepidopteran pheromone receptors were used in 3′ RACE reactions to identify candidate pheromone receptors expressed in the antennae of female navel orangeworm. Two near full-length transcripts of 1469 and 1302 nt encoding the complete open reading frames for proteins of 446 and 425 amino acids, respectively, were identified. Based on BLAST homology and phylogenetic analyses, the putative proteins encoded by these transcripts are members of the lepidopteran pheromone receptor subfamily. Characterization of these transcripts indicates that they are alternatively spliced products of a single gene. Tissue expression studies indicate that the transcripts are female-biased with detection mainly in female antennae. To the best of our knowledge, these transcripts represent the first detection of alternatively spliced female-biased members of the lepidopteran pheromone receptor subfamily.

Keywords: odorant receptor, pheromone receptor subfamily, alternative splicing, navel orangeworm, 3′ RACE

Introduction

The insect chemosensory system is critical for the detection of chemical cues in the environment, processing these signals in the central nervous system and eliciting behavioral responses to these stimuli (Smith, 2007). Within the chemosensory system, olfaction plays an important role in mate and host plant seeking behaviors (Depetris-Chauvin et al., 2015). Two major molecular components of the olfactory system are odorant binding proteins, which serve as a link between the external environment to shuttle hydrophobic volatile compounds through the sensillar lymph, and odorant receptors (ORs), which serve as key detectors of olfactants present in the environment and when bound by ligand, play a role in transducing signals along the olfactory neuron (Leal, 2013). ORs are part of a diverse family of seven transmembrane domain proteins, which are located on the

dendritic membranes of olfactory neurons. The first insect ORs were identified from the *Drosophila* genome (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999) and their structure is quite different from their mammalian counterparts (Bargmann, 2006; Benton, 2006). Because ORs show poor sequence homologies both within and between species, they have been identified mainly through bioinformatic algorithms to scan completed genomes or transcriptome sequences.

For the Lepidoptera, the first ORs were identified from *Bombyx mori* and *Heliothis virescens* through analysis of sequenced genomes (Krieger et al., 2004, 2005; Sakurai et al., 2004; Nakagawa et al., 2005). Further mining of the *B. mori* genome led to the identification of 48 ORs, and a subfamily, grouped by relatedness, contained putative pheromone receptors from both *B.mori* and *H.virescens* (Wanner et al., 2007). Subsequently, members of this subfamily have been referred to as male-biased sex pheromone receptor clade (Jordan et al., 2009), sex pheromone receptor subfamily (Miura et al., 2009, 2010; Wanner et al., 2010), pheromone receptors (Patch et al., 2009; Große-Wilde et al., 2010), and male-specific OR subfamily (Mitsuno et al., 2008). To gain a better understanding of how lepidopteran sex pheromones work at the molecular level, research to identify and characterize pheromone receptors in males has been the topic of much research (Krieger et al., 2004, 2005; Sakurai et al., 2004; Nakagawa et al., 2005; Große-Wilde et al., 2007, 2010; Wanner et al., 2007, 2010; Mitsuno et al., 2008; Jordan et al., 2009; Miura et al., 2009, 2010; Patch et al., 2009; Xu et al., 2012). However, some members of the pheromone receptor subfamily are also activated by plant kairomones (Jordan et al., 2009; Bengtsson et al., 2014), indicating that not all of these ORs respond to pheromones. Because of the importance of male sex pheromone receptors in enhancing mating disruption in lepidopteran pest control programs, much research has gone into their discovery and characterization, however, female attractants and their ORs remain an untapped resource for future lepidopteran control efforts.

The navel orangeworm, *Amyelois transitella* Walker (Lepidoptera:Pyralidae), is the major insect pest of almonds and pistachios in California, as well as a pest of other crops including walnuts and figures. Control of navel orangeworm is mainly achieved with sanitation techniques and by using pyrethroids and insect growth regulators, but other control methods using sex pheromones for mating disruption, and female attractants or oviposition attractants are sorely needed (Higbee et al., 2014). Recently, two pheromone subfamily receptors from navel orangeworm males have been identified and characterized (Xu et al., 2012). In this current study, we describe the identification of two transcripts expressed in antennae of navel orangeworm females that encode ORs with high similarity to those belonging to the lepidopteran pheromone receptor subfamily. A degenerate primer approach was used to identify the expressed transcripts (Garczynski et al., 2012) and the cDNAs encoding the full open reading frames were obtained using 5′RACE. Comparison of these sequences indicated that they may be produced by alternative splicing. Alternative splicing is a mechanism by which multiple mRNAs can be produced from a single gene, thereby increasing an organism's proteome (Nilsen and Graveley, 2010; Kornblihtt et al., 2013). To characterize the navel orangeworm transcripts, we included comparison of the cDNA transcript sequences to a partial cloned gene sequence and determined their tissue expression profiles. Implications of these results are discussed.

Materials and Methods

Insects and Dissection

Male and female pupae were obtained from Bradley Higbee, Research Entomologist (Paramount Farming Co., Bakersfield, CA) and antennae were dissected from 50 adults within 24 h of emergence and placed directly into 1.5 ml microfuge tubes containing 100 µl RNAlater® (Ambion, Austin, TX). Antennae in RNAlater® were stored for up to 4 weeks at 4°C until RNA was extracted (see below). Insects for expression studies were obtained from a lab colony maintained at University of California, Davis. For expression studies, antennae, heads, thoraces, and abdomens were dissected from newly eclosed adults (0–24 h old), placed into tubes containing 100 µl RNAlater® and stored at 4°C until the RNA was extracted.

RNA Extraction, cDNA Synthesis, Genomic DNA Extraction

Total RNA was extracted from dissected antennae, heads, thoraces and abdomens using the RNeasy® Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol for purification of total RNA from animal tissues. SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA) was used to generate cDNA from total RNA extracted from male and female antennae using the CDSIII/3′PCR primer and SMART II™ A oligonucleotide (Clontech, Mountain View, CA). Double-strand (ds) cDNAs and cDNA amplification were performed using components and procedures of the SMART™ PCR cDNA Synthesis Kit (Clontech, Mountain View, CA). Amplified cDNAs were purified using the High Pure PCR Product Purification Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Genomic DNA was prepared from adult male and female abdomens using the DNeasy® Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol for purification of total DNA from animal tissue. Total DNA was quantitated using the Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

Identification of Pheromone Receptors by 3′ RACE Degenerate Primer PCR

Identification of putative members of the pheromone receptor family was achieved by amplifying cDNA using degenerate primers designed against a conserved amino acid region in the C-terminus of lepidopteran pheromone receptors and a 3′Rapid amplification of cDNA ends (RACE) protocol as previously described (Garczynski et al., 2012). PCR reactions were set up using amplified antennal cDNA as template and the forward primer PR0 (5′-GTNCCNTGGGARTAYATGGAYAC-3′; Garczynski et al., 2012) and CDSIII/3′PCR as the reverse primer using the following conditions: initial denaturation for

5 min at 94°C, then amplification for 30 s at 94°C, 30 s at 62°C, 3 min at 72°C for 40 cycles, followed by a final 5 min 72°C incubation. PCR products were separated on 1.2% agarose gels containing ethidium bromide and visualized on a UV light box. Excised bands were extracted and purified using GenElute minus EtBr spin columns (Sigma, St. Louis, MO), and the purified PCR products were cloned using the TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA) with TOP 10 *Escherichia coli* chemically competent cells. Plasmid DNA was extracted from picked colonies using the QIAprep spin mini prep kit (Qiagen, Valencia, CA). The cDNA clones were sequenced at MC Laboratories (MCLab, San Francisco, CA).

Cloning Full Length Receptor Transcripts

To amplify the 5′ end of the female ORs identified above, a 5′RACE protocol was performed using the SMART™ RACE cDNA amplification kit (Clontech, Mountain View, CA) according to manufacturer supplied protocols. For 5′RACE, first strand cDNA was prepared as above except that total RNA was converted to cDNA using the 5′-RACE CDS Primer A and SMART II™ A oligonucleotide (Clontech, Mountain View, CA). From the cDNA sequences obtained above, reverse primers were designed for use in 5′RACE reactions using the Primer3 (v 0.4.0; http://frodo.wi.mit.edu/primer3/). Amplification was performed using the 10X Universal Primer A Mix as forward primer and sequence specific primers (AtraOR4 5′RACE Rev1: 5′- GAAAAGTGAAATACGACGGCGATG-3′ and AtraOR4 5′RACE Rev2: 5′- CCATATCCGTAACTCCGAGAGCC-3′) and the following conditions; initial denaturation for 3 min at 94°C, then amplification for 30 s at 94°C, 2 min at 72°C for 40 cycles, followed by a final 5 min 72°C incubation. PCR products were separated on 1% agarose gels and excised, TA cloned and sequenced as above. To obtain the full length sequences of the open reading frames for each receptor, PCR reactions were done with sequence specific primers (AtraOR4 ORF Fwd: 5′-GTATTTAAAATGGATATCAGTGCACAAAAT AGAGC -3′ and AtraOR4 ORF Rev: 5′- CTACTTGCATAA ATTAATCAATTTTTCGTAGAAAAGTG-3′) and the reaction conditions above. PCR products were cloned and sequenced as above, and the consensus sequence of three clones was generated in Geneious (version 7.1.9 created by Biomatters, available from http://www.geneious.com/). To determine identity of encoded proteins, tblastn on the National Center for Biotechnology Information website was used (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Classification of the AtraOR4 Receptors

Phylogenetic and evolutionary analyses were conducted using MEGA5 software (Tamura et al., 2011) to classify the AtraOR4 pheromone subfamily receptors. Amino acid sequences for ORs identified from *B. mori* (Wanner et al., 2007) and *H. virescens* (Krieger et al., 2002, 2004) along with odorant receptor members of the pheromone receptor subfamily from *Ostrinia nubialis* (Wanner et al., 2010), *Manduca sexta* (Patch et al., 2009), *Cydia pomonella* (Bengtsson et al., 2012; Garczynski, unpublished), *Plutella xylostella* (Mitsuno et al., 2008), *Mythimna separata* (Mitsuno et al., 2008), *Diaphania indica* (Mitsuno et al., 2008), and *A. transitella* (this study) were aligned using the Clustal W program (Thompson et al., 1994) built into the MEGA5 software. The bootstrap consensus tree was inferred from 500 replicates (Felsenstein, 1985) and branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 78 amino acid sequences. All positions with less than 50% site coverage were eliminated. That is, fewer than 50% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 406 positions in the final dataset.

Amplification of AtraOR4 Gene

To amplify the 3′ end of the *AtraOR4* gene, gene specific primers derived from cDNA sequences were used (AtraOR4 3′UTR Fwd1: 5′- GAACAGGGAGAATCGATTCATCG-3′ and AtraOR4 ORF Rev: see above; 200 nM final concentration) in PCR reactions with genomic DNA as template and using Advantage Taq polymerase (Clontech). PCR reactions were carried out using the following conditions; initial denaturation for 3 min at 94°C, then amplification for 20 s at 94°C, 20 s at 62°C, 3 min at 72°C for 40 cycles, followed by a final 5 min 72°C incubation. PCR products were separated on 1% agarose gels and excised and TA cloned as described above. Resultant gene clones were sequenced and the consensus sequence of three clones was generated in Geneious (version 7.1.9 created by Biomatters, available from http://www.geneious.com/).

Analysis of Receptor Expression

To determine which tissues express AtraOR4, reverse transcription PCR (RT-PCR) was used. Total RNA was extracted from antennae, heads, thoraces, and abdomens as above, and quantitated using the Quant-iT™ RiboGreen® RNA assay kit (Invitrogen, Carlsbad, CA). First strand cDNA synthesis was performed as above, using 100 ng of total RNA and primed with 5′-RACE CDS Primer A. PCR amplification was done using gene specific primers (200 nM final concentration) to detect AtraOR4 (AtraOR4 3′UTR Fwd1 and AtraOR4 ORF Rev; sequences are above), AtraOrco (AtraOrco Fwd: 5′- AGATGTTGGCTCGTTCTGCT-3′ and AtraOrco Rev: 5′- AAGCCGCTTCCATTACTGAC-3′) and Actin (Actin 1F: 5′- GGTCGCGATCTCACAGACTA-3′ and Actin 1R: 5′-TCGAGTTGTAGGTGGTTTCG-3′), and cDNA template equivalent to 5 ng of input RNA using the following conditions; initial denaturation for 3 min at 94°C, then amplification for 20 s at 94°C, 20 s at 62°C, 30 s at 72°C for 35 cycles, followed by a final 5 min 72°C incubation. PCR products were separated by loading 1/4th of the total reaction onto 1.5% agarose gels and visualized on a UV light box. To confirm identity of

PCR products, bands were excised, TA cloned and sequenced as above.

Results

Analysis of cDNA and Deduced Amino Acid Sequences of AtraOR4 Transcripts

To detect potential pheromone receptor subfamily members expressed in the antennae of female navel orangeworm, degenerate primers and a 3′ RACE technique was used (Garczynski et al., 2012). The 3′ end of cDNAs of transcripts present in female antennae were amplified and PCR products visualized on agarose gels were excised and TA cloned (data not shown). The cDNA sequence of one clone encoded for a putative pheromone receptor subfamily member (named AtraOR4) based on homology of TBLASTN searches using the deduced amino acid sequence against those present in the NCBI non-redundant nucleotide database (data not shown).

The cDNA sequence obtained by 5′ RACE encompassed the putative start methionine of AtraOR4 (data not shown). An oligonucleotide primer encompassing the nucleotides surrounding the start methionine was designed and used in a 3′ RACE reaction to amplify the cDNA encoding the full open reading frame. Two PCR products (∼1550 and 1400 bp) were visualized on agarose gels (**Figure 1A**), excised and TA cloned. The nucleotide sequences of the PCR products were determined, yielding actual transcript lengths of 1469 (GenBank ID: JN701807) and 1302 nt (GenBank ID: JN701806) encoding for 446 and 425 amino acids (Figure S1B and Figure S1A, respectively). The 1302 nt transcript was designated AtraOR4 and the 1469 nt transcript, AtraOR4A. Alignment of the nucleotide sequences of these transcripts showed that they were nearly identical except for an additional 167 nt internal sequence in the 3′ end of the longer AtraOR4A transcript (Figure S2, Supplementary Material). Alignment of the deduced amino acid sequences revealed that the two putative proteins are nearly identical for the first 349 amino acids, and then diverge at the C-terminus (Figure S3, Supplementary Material).

To determine if AtraOR4 and AtraOR4A are putative members of the lepidopteran pheromone receptor subfamily, TBLASTN searches (Altschul et al., 1990) were done using the deduced amino acid sequences against the NCBI non-redundant nucleotide database limited to arthropods. AtraOR4 and AtraOR4A displayed 34–41% identity with the top 15 published blast hits (*E*-values ranging from 1.00^{-77} to 2.00^{-105}) and all of the similar sequences were members of the pheromone receptor subfamily (data not shown). These results indicate that on the basis of amino acid sequence homology, AtraOR4, and AtraOR4A are members of the lepidopteran pheromone receptor subfamily. To confirm these results, a phylogenetic

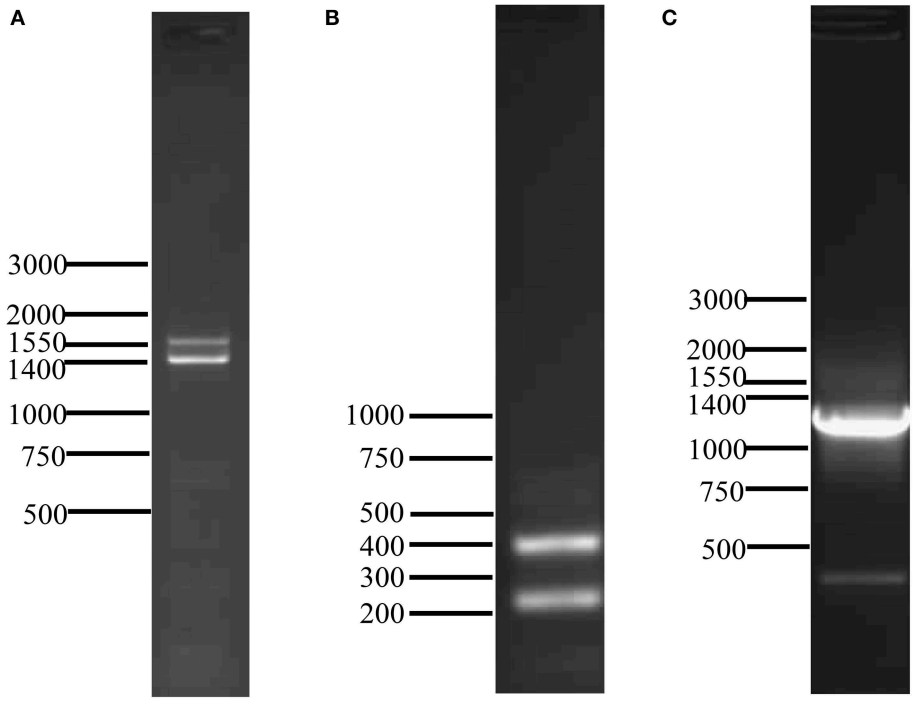

FIGURE 1 | PCR detection of AtraOR4 and AtraOR4A transcripts and 3′ end of AtraOR4 gene. (A) Full-length transcripts amplified by 3′ RACE. Amplification products were generated by 3′ RACE and visualized on 1% agarose gels by ethidium bromide staining and UV illumination. **(B)** RT-PCR detection of the 3′ end of AtraOR4 and AtraOR4A transcripts. Amplification using RT-PCR and transcript specific primers. PCR products were visualized on 1.5% agarose gels by ethidium bromide staining and UV illumination. **(C)** PCR detection of the 3′ end of AtraOR4 gene. Amplification was done using transcript specific primers and PCR product was visualized on 1% agarose gels by ethidium bromide staining and UV illumination.

analysis was performed and a tree was constructed. The amino acid sequences of 78 lepidopteran ORs, including 26 putative pheromone subfamily receptors, were aligned using ClustalW and the resulting Neighbor Joining tree shows that AtraOR4 and AtraOR4A cluster within the pheromone receptor subfamily (**Figure 2**).

Analysis of the 3′ Region of AtraOR4 Transcripts and AtraOR4 Gene

To further analyze the 3′ regions of the two nearly identical transcripts encoding AtraOR4 and AtraOR4A, specific oligonucleotide primers upstream of the divergent 3′ nucleotide sequences were designed for use in 3′ RACE. Consistent with the results above (**Figure 1A**), two PCR products of approximately 250 and 400 nt were detected in the 3′ RACE reaction performed with the transcript specific primers (**Figure 1B**). The DNA sequence of these products indicated, as above, that they are nearly identical except for an additional 167 nt of internal sequence in the AtraOR4A product (Figure S2, Supplementary Material). Because the dinucleotide AG was at the end of the additional 167 nt internal sequence and directly preceded the end of the shorter transcript we considered the possibility that these two transcripts were alternatively spliced products of the same gene. To test this, oligonucleotide primers encompassing the 3′ end of the transcripts were used in PCR reactions to amplify genomic DNA. A single product of approximately 1300 bp was detected on agarose gels (**Figure 1C**) from the PCR amplification using the transcript specific primers. These results indicated that the AtraOR4 and AtraOR4A transcripts were produced from the same gene.

To determine if the AtraOR4 and AtraOR4A transcripts were generated from the same gene, the PCR product of the genomic DNA amplification was cloned and sequenced. The cloned gene product was 1271 bp (**Figure 3**; GenBank ID: JN701808). Alignment of the 3′ regions of the AtraOR4 and AtraOR4A transcripts with the genomic DNA showed that their sequences are identical and contained within the gene sequence (**Figure 3**). The AtraOR4 gene sequence contains an intron with a canonical dinucleotide GT splice site shared by both the AtraOR4 and AtraOR4A transcripts. For AtraOR4A, a canonical dinucleotide AG splice site is located 931 bp downstream yielding the 3′ end of its transcript and a canonical dinucleotide AG splice site is located 1098 bp downstream for the AtraOR4 transcript (**Figure 3**).

Tissue Expression Profile of AtraOR4

To determine where the AtraOR4 and AtraOR4A transcripts are expressed in adult males and females, a tissue expression profile using RT-PCR was performed with cDNA prepared from total RNA extracted from dissected antennae, heads (without antennae), thoraces, and abdomens (**Figure 4**). Expression of AtraOR4 and AtraOR4A was female-biased, with PCR products detected mainly in female antennae (**Figure 4**, top). AtraOrco, a conserved co-receptor that forms heteromers with ORs in insects (Larsson et al., 2004; Orco has previously been called OR83b in *Drosophila*, OR2 in lepidopterans and OR7 in mosquitoes), expression was detected in male antennae and to a lesser extent in heads, and in female antennae and to a lesser extent in heads and abdomens (**Figure 4**, middle). As a control, primers to detect AtraActin were used and it was detected in all tissues (**Figure 4**, bottom).

Discussion

Using degenerate oligonucleotide primers and 3′ RACE (Garczynski et al., 2012), we have identified two transcripts expressed in antennae of female navel orangeworm that encode putative members of the lepidopteran pheromone receptor subfamily. Analysis of cDNAs containing full-length ORFs indicate that these transcripts appear to be the products of alternative splicing from a single gene. Comparison of the transcript nucleotide sequences with genomic DNA sequence support that AtraOR4 and AtraOR4A are produced from the same gene by alternative splicing. Phylogenetic analysis supports that the proteins encoded by the AtraOR4 and AtraOR4A transcripts are members of the lepidopteran pheromone receptor subfamily. Tissue expression profiles indicate that these transcripts are mainly found in female antennae. While female-biased ORs have been previously identified in *B. mori* (Wanner et al., 2007; Anderson et al., 2009), these ORs were not homologous to members of the lepidopteran pheromone receptor subfamily. We believe AtraOR4 and AtraOR4A to be the first example of alternatively spliced, female-biased members of the lepidopteran pheromone receptor subfamily.

Alternative splicing is a common mechanism used to produce multiple proteins from a single gene and this process can significantly increase the size of an organism's proteome. For mammalian ORs, alternative splicing is extensive, but rarely occurs within the coding region (Volz et al., 2003; Young et al., 2003). Alternative splicing at the N-termini of ORs and mainly, gustatory receptors has been detected for *Drosophila*, *Anopheles*, *Aedes,* and *Tribolium* (Clyne et al., 2000; Hill et al., 2002; Robertson et al., 2003; Abdel-Latief, 2007; Kent et al., 2008), however, this is the first evidence of alternative splicing changing the C-terminal amino acid sequence in an OR from insects. The extent of alternative splicing of insect OR genes is unknown. Because most lepidopteran (and other insects) ORs are being identified from *de novo* assembled transcriptomes, detection of alternate spliced products may be difficult because of the limitations of bioinformatic resources (Florea, 2006). Until bioinformatics resources become sufficient enough to assemble and predict alternative spliced products, traditional methods of sequencing RACE amplified transcripts can still be used to identify alternately spliced OR gene products.

The predicted protein products for AtraOR4 and AtraOR4A align most closely with members of the pheromone receptor subfamily. In this current study, we did not attempt to determine odorant ligands for the protein products encoded by the AtraOR4 and AtraOR4A transcripts, but we expect that they will be either pheromone(s) or host-plant volatile(s) in nature. For some lepidopteran pheromone receptor subfamily members, the ligands for these ORs are sex pheromones (for examples see Sakurai et al., 2004; Wanner et al., 2010; Xu et al., 2012 among others). However, not all pheromone receptor subfamily members respond to sex pheromones. For *B. mori*, odorants that activate only OR1 (bombykol) and OR3 (bombykal) have

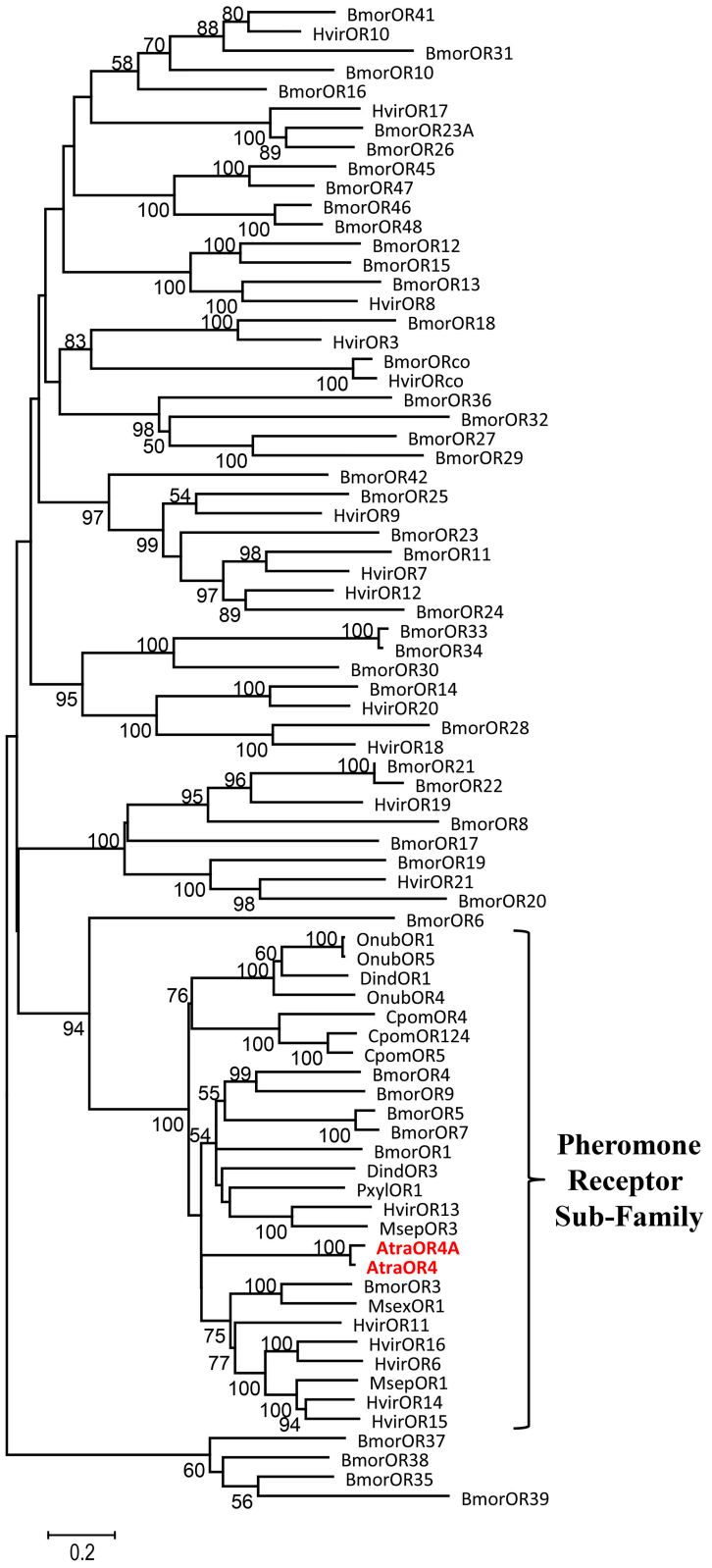

FIGURE 2 | Neighbor joining tree of AtraOR4 and AtraOR4A with 78 lepidopteran ORs, including 26 putative pheromone subfamily receptors from the following insects: *Bombyx mori* (Bmor), *Heliothis virescens* (Hvir), *Ostrinia nubialis* (Onub), *Manduca sexta* (Msex), *Cydia pomonella* (Cpom), *Plutella xylostella* (Pxyl), *Mythimna separata* (Msep), *Diaphania indica* (Dind), and *Amyelois transitella* (Atra).

```
AtraOR4gen     1 GAACAGGGAGAATCGATTCATCGTGCTAATTCTACTGATGAACGTCCAGAAACCGGTGAGGGTGAAGGCTCTC
AtraOR4A         GAACAGGGAGAATCGATTCATCGTGCTAATTCTACTGATGAACGTCCAGAAACCGGTGAGGGTGAAGGCTCTC
AtraOR4          GAACAGGGAGAATCGATTCATCGTGCTAATTCTACTGATGAACGTCCAGAAACCGGTGAGGGTGAAGGCTCTC

AtraOR4gen    74 GGAGTTACGGATATGGGTGTGGCTACTGTCGCTTCTGTAAGTAACATACGTTAATTATACATAAATAAATATA
AtraOR4A         GGAGTTACGGATATGGGTGTGGCTACTGTCGCTTCT
AtraOR4          GGAGTTACGGATATGGGTGTGGCTACTGTCGCTTCT

AtraOR4gen   147 AATAAAAATAAATATATACGGGACAAATAACACAGATTGAGTTAGCCTCGAAGTAAGACTGAAACTTGTGTTA
             220 CGATGTTACTAACTCAACGATACTATATTTTATAATAAATACTTATATAGATAAACATCCAAGACCCAGGCCA
             293 ATCAGAGAAAGTTCGTTTCTCATCATGCCCTAGCCGGGATTCGAACCCGGCACCTCCGGTGACACAGACAAGC
             366 GCACTACCGCTGCGCCACAGAGGCCGTCTACATACATACATACATATCATCACGTCTATATCCCTTGCGGAGT
             439 AAACAGAGCCAACAGACTGGAAAACACTGAAAAGCTACGTTCAGCTGTTTGGCTTAATGTTAGAATTGAGATT
             512 CAAATAAAGAGAGGTTGCTTGCTTATCGCCTAAAAGAAGAATCTCAAGTTTGTAAGCCAATCCCTTAGTCCCT
             585 TTTACGTCATCCATGGAAAGGAGATGGCATAGTCCTATTCTTTTTTCTGTTGGTGCCGGGAAACACACGGCAC
             658 GATTAATGTTACAAAAGATGTTACTAGTAATTTTCCAGAATAACACATCAGACTTATTTGGGCATACAACCAT
             731 TATAACGATAGACAAGATACATAGATTATGATCATCACACCTCTGGAAACGGCTCCCATTTGATGAATTTATT
             804 TGGTTAACATTTCTTTTTTCTTTCTTTCTTCCTCACCCTTGGCGTTAATCCTAGTCGCGTCCTCAGAGAGTAA
             877 CTCTAAATGCGCCAATGATCACAATTTAAGGAGTTATGTACTAATATTATGCCTTGTGGTTCCCAGCACCAAT
             950 ACAAAAAGAATAGACCAAACCATATCTTTCCCATGGATGTCGTAAAAGGCGACTAAGGGATAGGCTTATAAAA

AtraOR4gen 1023 TTGCGATTCTTTTTTTAGGCGATGGGCTGGCAACCTGTCACTATTTGGATCTCAATTCCATCATTAAGCCGAG
AtraOR4A                       GCGATGGGCTGGCAACCTGTCACTATTTGGATCTCAATTCCATCATTAAGCCGAG

AtraOR4gen 1096 CAGCTGAACGTGGCCATTCAGTCTTTTCGGGACTAATGGTATATGTATGTATGTACTAAAAACTTAATCCCGC
AtraOR4A        CAGCTGAGCGTGGCCATTCAGTCTTTTCGGGACTAATGGTATATGTATGTATGTACTAAAAACTTAATCCCGC

AtraOR4gen 1169 TTATACTTGTTGCTTAAAATTTGTTATTAATTATTCCAGATAATAAAAACATCGCTGTCGTATTTCACTTTTC
AtraOR4A        TTATACTTGTTGCTTAAAATTTGTTATTAATTATTCCAGATAATAAAAACATCGCTGTCGTATTTCACTTTTC
AtraOR4                                                ATAATAAAAACATCGCTGTCGTATTTCACTTTTC

AtraOR4gen 1242 TACGAAAAATTGATTAATTTATGCAAGTAG
AtraOR4A        TACGAAAAATTGATTAATTTATGCAAGTAG
AtraOR4         TACGAAAAATTGATTAATTTATGCAAGTAG
```

FIGURE 3 | Partial genomic DNA sequence encoding 3′ end of AtraOR4 and alignment of AtraOR4 and AtraOR4A transcripts. AtraOR4 genomic DNA sequence is in black, AtraOR4A is in red, and AtraOR4 is in blue. Putative splice sites are GT for excision and AG insertion.

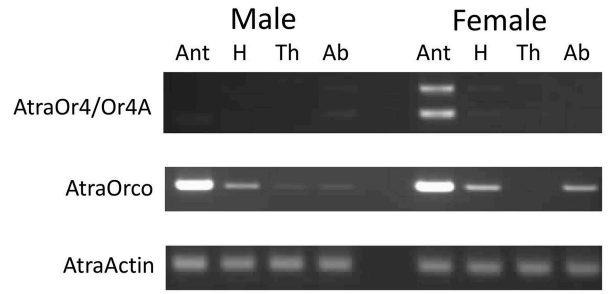

FIGURE 4 | AtraOR4 and AtraOR4A expression profile in cDNA prepared from total RNA extracted from male and female antennae (Ant), heads (H), thoraces (Th) and abdomens (Ab). Top: Detection of AtraOR4 and AtraOR4A using transcript specific primers in reverse transcription PCR (RT-PCR) reactions. **Middle**: Detection of AtraOrco using specific primers in RT-PCR reactions. **Bottom**: Detection of AtraActin using specific primers in RT-PCR reactions. For each panel, PCR products were visualized on 1.5% agarose gels by ethidium bromide staining and UV illumination. Bands produced were of the size predicted for the transcript specific primer pairs.

been identified while OR4, OR5, and OR6 remain orphans (Nakagawa et al., 2005). Furthermore, two ORs belonging to the pheromone receptor subfamily clade are activated by plant volatiles. In the light brown apple moth, *Epiphyas postvittana*, a pheromone receptor subfamily member, EpOR1, is stimulated by several plant volatiles with the strongest binding to methyl salicylate (1.8×10^{-12} M) and geraniol (5.8×10^{-11} M) (Jordan et al., 2009). More recently, a codling moth, *C. pomonella*, pheromone receptor subfamily member, CpomOR3, was shown to be activated by pear ester, an important host plant kairomone for this insect (Bengtsson et al., 2014). Perhaps the categorization of the pheromone receptor subfamily (aka male-biased sex pheromone receptor clade, sex pheromone receptor subfamily, pheromone receptors, and male-specific OR subfamily) should be reconsidered until a significant number of receptors has been de-orphanized.

There is not much information on the structure/function relationships of ligand binding for insect ORs. Much work is being done to determine which ligands activate different ORs, but this line of research does not tell us where on the receptors the odorants are binding. Recently, it has been determined that the C-terminal amino acids are important for odor specificity, namely conferring enantiomeric selectivity (Hill et al., 2015). Because alternate spliced products of AtraOR4 and AtraOR4A results in changes of the C-terminal amino acids of these proteins, it is possible that these changes enable these receptors to

recognize different ligands or discriminate different enantiomers of their ligands. However, until further characterizations of ORs are completed the role of the C-terminal amino acids in ligand binding remains speculative.

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Author Contributions

SG and WL conceived and designed the work, and supplied materials. SG performed the work and wrote the manuscript. WL and SG revised the manuscript to its final submitted form.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2015.00115

References

Abdel-Latief, M. (2007). A family of chemoreceptors in *Tribolium castaneum* (Tenebrionidae: Coleoptera). *PLoS ONE* 2:e1319. doi: 10.1371/journal.pone.0001319

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Anderson, A. R., Wanner, K. W., Trowell, S. C., Warr, C. G., Jaquin-Joly, E., Zagatti, P., et al. (2009). Molecular basis of female-specific odorant responses in *Bombyx mori*. *Ins. Biochem. Mol. Biol.* 39, 189–197. doi: 10.1016/j.ibmb.2008.11.002

Bargmann, C. I. (2006). Comparative chemosensation from receptors to ecology. *Nature* 444, 295–301. doi: 10.1038/nature05402

Bengtsson, J. M., Gonzalez, F., Cattaneo, A. M., Montagne, N., Walker, W. B., Bengtsson, M., et al. (2014). A predicted sex pheromone receptor of codling moth *Cydia pomonella* detects the plant volatile pear ester. *Front. Ecol. Evol.* 2:33. doi: 10.3389/fevo.2014.00033

Bengtsson, J. M., Trona, F., Montagne, N., Anfora, G., Ignell, R., Witzgall, P., et al. (2012). Putative chemosensory receptors of the codling moth, *Cydia pomonella*, identified by antennal transcriptome analysis. *PLoS ONE* 7:e31620. doi: 10.1371/journal.pone.0031620

Benton, R. (2006). On the ORigin of smell: odorant receptors in insects. *Cell. Mol. Life Sci.* 63, 1579–1585. doi: 10.1007/s00018-006-6130-7

Clyne, P. J., Warr, C. G., Freeman, M. R., Lessing, D., Kim, J., and Carlson, J. R. (1999). A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron* 22, 327–338.

Clyne, P. J., Warr, C. G., and Carlson, J. R. (2000). Candidate taste receptors in *Drosophila*. *Science* 287, 1830–1834. doi: 10.1126/science.287.5459.1830

Depetris-Chauvin, A., Galagovsky, D., and Grosjean, Y. (2015). Chemicals and chemoreceptors: ecologically relevant signals driving behavior in *Drosophila*. *Front. Ecol. Evol.* 3:41. doi: 10.3389/fevo.2015.00041

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791. doi: 10.2307/2408678

Florea, L. (2006). Bioinformatics of alternative splicing and its regulation. *Brief. Bioinformatics*. 7, 55–69. doi: 10.1093/bib/bbk005

Gao, Q., and Chess, A. (1999). Identification of candidate *Drosophila* olfactory receptors from genomic DNA. *Genomics* 60, 31–39.

Garczynski, S. F., Wanner, K. W., and Unruh, T. R. (2012). Identification and initial characterization of the 3′ end of gene transcripts encoding putative members of the pheromone receptor subfamily in Lepidoptera. *Insect Sci.* 19, 64–74. doi: 10.1111/j.1744-7917.2011.01423.x

Große-Wilde, E., Gohl, T., Bouché, E., Breer, H., and Krieger, J. (2007). Candidate pheromone receptors provide the basis for the response of distinct antennal neurons to pheromone compounds. *Eur. J. Neurosci.* 25, 2364–2373. doi: 10.1111/j.1460-9568.2007.05512.x

Große-Wilde, E., Stieber, R., Forstner, M., Krieger, J., Wicher, D., and Hansson, B. S. (2010). Sex-specific odorant receptors of the tobacco hornworm *Manduca sexta*. *Front. Cell. Neurosci.* 4:22. doi: 10.3389/fncel.2010.00022

Higbee, B. S., Burks, C. S., and Larsen, T. E. (2014). Demonstration and characterization of a persistent pheromone lure for the navel orangeworm, *Amyelois transitella* (Lepidoptera: Pyralidae). *Insects* 5, 596–608. doi: 10.3390/insects5030596

Hill, C. A., Fox, A. N., Pitts, R. J., Kent, L. B., Tan, P. L., Chrustal, M. A., et al. (2002). G protein-couple receptors in *Anopheles gambiae*. *Science* 298, 176–178. doi: 10.1126/science.1076196

Hill, S. R., Majeed, S., and Ignell, R. (2015). Molecular basis for odorant receptor tuning: a short C-terminal sequence is necessary and sufficient for selectivity of mosquito OR8. *Insect Mol. Biol.* 24, 491–501. doi: 10.1111/imb.12176

Jordan, M. D., Anderson, A., Begum, D., Carraher, C., Authier, A., Marshall, S. D. G., et al. (2009). Odorant receptors from the light brown apple moth (*Epiphyas postvittana*) recognize important volatile compounds produced by plants. *Chem. Senses* 34, 383–394. doi: 10.1093/chemse/bjp010

Kent, L. B., Walden, K. K. O., and Robertson, H. M. (2008). The Gr family of candidate gustatory and olfactory receptors in the yellow-fever mosquito *Aedes aegypti*. *Chem. Senses* 33, 79–93. doi: 10.1093/chemse/bjm067

Kornblihtt, A. R., Schor, I. E., Allo, M., Dujardin, G., Petrillo, E., and Munoz, M. J. (2013). Alternative splicing: a pivotal step between eukaryotic transcription and translation. *Nat. Rev. Mol. Cell Biol.* 13, 153–165. doi: 10.1038/nrm3525

Krieger, J., Große-Wilde, E., Gohl, T., and Breer, H. (2005). Candidate pheromone receptors of the silkmoth *Bombyx mori*. *Eur. J. Neurosci.* 21, 2167–2176. doi: 10.1111/j.1460-9568.2005.04058.x

Krieger, J., Große-Wilde, E., Gohl, T., Dewer, Y. M. E., Raming, K., and Breer, H. (2004). Genes encoding candidate pheromone receptors in a moth (*Heliothis virescens*). *Proc. Nat. Acad. Sci. U.S.A.* 101, 11845–11850. doi: 10.1073/pnas.0403052101

Krieger, J., Raming, K., Dewer, Y. M. E., Bette, S., Conzelmann, S., and Breer, H. (2002). A divergent gene family encoding candidate olfactory receptors of the moth *Heliothis virescens*. *Eur. J. Neurosci.* 16, 619–628. doi: 10.1046/j.1460-9568.2002.02109.x

Larsson, M. C., Domingos, A. I., Jones, W. D., Chiappe, M. E., Amrein, H., and Vosshall, L. B. (2004). Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* 43, 703–714. doi: 10.1016/j.neuron.2004.08.019

Leal, W. S. (2013). Odorant Reception in insects: roles of receptors, binding proteins, and degrading enzymes. *Annu. Rev. Entomol.* 58, 373–391. doi: 10.1146/annurev-ento-120811-153635

Mitsuno, H., Sakurai, T., Murai, M., Yasuda, T., Kugimiya, S., Ozawa, R., et al. (2008). Identification of receptors of main sex-pheromone components of three lepidopteran species. *Eur. J. Neurosci.* 28, 893–902. doi: 10.1111/j.1460-9568.2008.06429.x

Miura, N., Nakagawa, T., Tatsuki, S., Touhara, K., and Ishikawa, Y. (2009). A male-specific odorant receptor conserved through the evolution of sex pheromones in *Ostrinia* moth species. *Int. J. Biol. Sci.* 5, 319–330. doi: 10.7150/ijbs.5.319

Miura, N., Nakagawa, T., Touhara, K., and Ishikawa, Y. (2010). Broadly and narrowly tuned odorant receptors are involved in female sex pheromone reception in *Ostrinia* moths. *In. Biochem. Mol. Biol.* 40, 64–73. doi: 10.1016/j.ibmb.2009.12.011

Nakagawa, T., Sakurai, T., Nishioka, T., and Touhara, K. (2005). Insect sex-pheromone signals mediated by specific combinations of olfactory receptors. *Science* 307, 1638–1642. doi: 10.1126/science.1106267

Nilsen, T. W., and Graveley, B. R. (2010). Expansion of the eukaryotic proteome by alternative splicing. *Nature* 463, 457–463. doi: 10.1038/nature08909

Patch, H. M., Velarde, R. A., Walden, K. K. O., and Robertson, H. M. (2009). A candidate pheromone receptor and two odorant receptors of the hawkmoth *Manduca sexta*. *Chem. Senses* 34, 305–316. doi: 10.1093/chemse/bjp002

Robertson, H. M., Warr, C. G., and Carlson, J. R. (2003). Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 100, 14537–14542. doi: 10.1073/pnas.2335847100

Sakurai, T., Nakagawa, T., Mitsuno, H., Mori, H., Endo, Y., Tanoue, S., et al. (2004). Identification and functional characterization of a sex pheromone receptor in the silkmoth *Bombyx mori*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16653–16658. doi: 10.1073/pnas.0407596101

Smith, D. P. (2007). Odor and pheromone detection in *Drosophila melanogaster*. *Pflügers Arch. Eur. J. Physiol.* 454, 749–758. doi: 10.1007/s00424-006-0190-2

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739. doi: 10.1093/molbev/msr121

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* 22, 4673–4680. doi: 10.1093/nar/22.22.4673

Volz, A., Ehlers, A., Younger, R., Forbes, S., Trowsdale, J., Schnorr, D., et al. (2003). Complex transcription and splicing of odorant receptor genes. *J. Biol. Chem.* 278, 19691–19701. doi: 10.1074/jbc.M212424200

Vosshall, L. B., Amrein, H., Morozov, P. S., Rzhetsky, A., and Axel, R. (1999). A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell* 96, 725–736.

Wanner, K. W., Anderson, A. R., Trowell, S. C., Theilmann, D. A., Robertson, H. M., and Newcomb, R. D. (2007). Female-biased expression of odourant receptor genes in the adult antennae of the silkworm, *Bombyx mori*. *In. Mol. Biol.* 16, 107–119. doi: 10.1111/j.1365-2583.2007.00708.x

Wanner, K. W., Nichols, A. S., Allen, J. E., Bunger, P. L., Garczynski, S. F., Linn, C. E., et al. (2010). Sex pheromone receptor specificity in the European corn borer moth, *Ostrinia nubilalis*. *PLoS ONE* 5:e8685. doi: 10.1371/journal.pone.0008685

Xu, P., Garczynski, S. F., Atungulu, E., Syed, Z., Choo, Y.-M., Vidal, D. M., et al. (2012). Moth sex pheromone receptors and deceitful parapheromones. *PLoS ONE* 7:e41653. doi: 10.1371/journal.pone.0041653

Young, J. M., Shykind, B. M., Lane, R. P., Tonnes-Priddy, L., Ross, J. A., Walker, M., et al. (2003). Odorant receptor expressed sequence tags demonstrate olfactory expression of over 400 genes, extensive alternate splicing and unequal expression levels. *Genome Biol.* 4:R71. doi: 10.1186/gb-2003-4-11-r71

Zuckerkandl, E., and Pauling, L. (1965). "Evolutionary divergence and convergence in proteins," in *Evolving Genes and Proteins*, eds V. Bryson and H. J. Vogel (New York, NY: Academic Press), 97–166.

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