Article

# IDENTIFICATION OF THE WESTERN TARNISHED PLANT BUG (*Lygus hesperus*) OLFACTORY CO-RECEPTOR ORCO: EXPRESSION PROFILE AND CONFIRMATION OF ATYPICAL MEMBRANE TOPOLOGY

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Lygus hesperus *(western tarnished plant bug) is an agronomically important pest species of numerous cropping systems. Similar to other insects, a critical component underlying behaviors is the perception and discrimination of olfactory cues. Consequently, the molecular basis of olfaction in this species is of interest. To begin to address this issue, we utilized homology-based PCR as a commonly accepted abbreviation but if necessary it is polymerase chain reaction methods to identify the* L. hesperus *olfactory receptor co-receptor (Orco) ortholog, a receptor that has been shown to be essential for olfaction. The* L. hesperus *Orco (LhOrco) shares significant sequence homology with known Orco proteins in other insects. Parallel experiments using the sympatric sister species,* Lygus lineolaris *(tarnished plant bug), revealed that the Lygus Orco gene was completely conserved. Surprisingly, a majority of the membrane topology prediction algorithms used in the study predicted LhOrco to have both the N and C terminus intracellular. In vitro immunofluorescent microscopy experiments designed to probe the membrane topology of transiently expressed LhOrco, however, refuted those predictions and confirmed that the*

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*protein adopts the inverted topology (intracellular N terminus and an extracellular C terminus) characteristic of Orco proteins. RT-PCR analyses indicated that LhOrco transcripts are predominantly expressed in adult antennae and to a lesser degree in traditionally nonolfactory chemosensory tissues of the proboscis and legs. Expression is not developmentally regulated because transcripts were detected in all nymphal stages as well as eggs. Taken together, the results suggest that LhOrco likely plays a critical role in mediating* L. hesperus *odorant perception and discrimination.*


**Keywords:** *Lygus hesperus*; olfactory co-receptor; olfaction; immunofluorescent microscopy; membrane topology

## INTRODUCTION

The western tarnished plant bug, *Lygus hesperus*, belongs to a large complex of morphologically similar *Lygus* plant bug species from the Miridae family that includes *Lygus lineolaris*, *L. elisus*, *L. shulli,* and *L. rugulipennis* among others (Schwartz and Foottit, 1998). They are an economically important polyphagous hemipteran pest that primarily cause damage by feeding on plant reproductive tissue resulting in deformation of developing fruit, reduced vegetative growth, and feeding site necrosis (Strong, 1970). *Lygus* spp. affect more than 150 host plants including traditional crops such as cotton, strawberries, and alfalfa (Scott, 1977; Wheeler, 2001) as well as emerging oilseed crops that have significant potential as biofuel feedstocks (Butts and Lamb, 1990; Turnock et al., 1995; Naranjo et al., 2008; Ritter et al., 2010; Naranjo et al., 2011).

Like most insects, *Lygus* spp. interactions with their environment are predominantly governed by chemical signals. Female *L. hesperus* have been shown to be strongly attracted to volatiles from flowering alfalfa that have sustained feeding damage (Blackmer et al., 2004; Blackmer and Cañas, 2005) as well as to volatiles from flowering lesquerella (Blackmer and Byers, 2009). Female *L. rugulipennis* (European tarnished plant bug) are likewise attracted to volatiles released by fava bean (*Vicia faba*) (Frati et al., 2008). Volatiles also trigger sex specific responses. Putative sex pheromone components of *L. rugulipennis* females have been shown to have an attractive effect on males in field tests (Innocenzi et al., 2005). These behavioral responses are strongly correlated with the antenneal olfactory system. Electrophysiological recordings of *L. lineolaris* antennal responses (electroantennograms; EAG) showed that insect-produced butyrates and plant-derived green leaf volatiles both triggered electrical activity (Chinta et al., 1994). Antennae in *L. rugulipennis* were likewise reported to respond to volatiles from damaged and nondamaged host plants (Frati et al., 2009) while male *L. rugulipennis* antennae were significantly more responsive than female antennae to putative sex pheromone components (Innocenzi et al., 2004). In *L. hesperus*, Williams et al. (2010) observed a correlation between EAG responses and behavioral responses for (*E, E*)-α-farnesene and (*E*)-β-ocimene, two plant volatiles emitted by alfalfa (i.e., host plant) in response to damage.

In insects, olfactory detection of chemical odorants involves the activation of odorant receptors (ORs) expressed within the olfactory sensory neurons of the antennae. Surprisingly, insect ORs has been shown to be mechanistically and structurally distinct from their mammalian counterparts (Ha and Smith, 2009; Nakagawa and Vosshall, 2009; Sato and Touhara, 2009; Kaupp, 2010). Similar to G protein-coupled receptors (GPCRs), insect

ORs contain seven transmembrane helices (TM). They, however, exhibit a unique membrane topology in which the N terminus is intracellular and the C terminus is extracellular (GPCRs exhibit the opposite topology) (Benton et al., 2006; Lundin et al., 2007; Smart et al., 2008; Tsitoura et al., 2010) and share no sequence homology with vertebrate ORs or vertebrate GPCRs (Wistrand et al., 2006). Insect ORs are frequently co-expressed with a nonconventional OR previously referred to as OR83b in *Drosophila melanogaster*, OR2 in *Bombyx mori*, and OR7 in mosquitoes, but which has recently been renamed olfactory receptor co-receptor (Orco) (Vosshall and Hansson, 2011). Unlike other insect ORs, which exhibit little sequence homology, Orco is strikingly well conserved across insect species (Sato and Touhara, 2009). Orco interacts with conventional ligand-specific ORs to form heterodimeric complexes that are critical for stabilization and trafficking of conventional ORs (Larsson et al., 2004; Benton et al., 2006). The OR-Orco complex forms a ligand-gated ion channel at the plasma membrane that opens in response to odorant binding with the odorant binding site likely residing in the conventional OR subunit (Nakagawa et al., 2005; Sato et al., 2008; Wicher et al., 2008; Nichols et al., 2011). Despite contributing little to odorant binding and/or discrimination, the Orco subunit is critical for ion channel formation (Larsson et al., 2004; Jones et al., 2011). Indeed, disruption of Orco expression in *Drosophila* significantly impairs odorant responses (Larsson et al., 2004). Consequently, identification of Orco genes in problematic insect species could facilitate the development of novel pest management strategies.

In *Lygus* spp., little is known at the molecular level of olfaction beyond a single protein termed *Lygus* antennal protein (LAP) (Dickens et al., 1995; Dickens et al., 1998; Vogt et al., 1999), which as an odorant binding protein is thought to mediate the movement of apolar chemical odorants through the aqueous antennae sensillum to the ORs housed within the olfactory sensory neurons (Rützler and Zwiebel, 2005; Pelosi et al., 2006). Nothing is known of *Lygus* spp. ORs. Furthermore, while Orco orthologs have been identified in lice (Kirkness et al., 2010) and aphids (Smadja et al., 2009) (members of the hemipteroid assemblage which also includes bugs), no bug Orco has been identified. Consequently, to gain a better understanding of olfaction within *L. hesperus* specifically and bugs in general, we sought to identify the *Lygus* Orco gene product and to determine its expression profile.

## MATERIALS AND METHODS

### *Insect Rearing*

*Lygus hesperus* were obtained from a laboratory colony maintained at the USDA-ARS Arid Land Agricultural Research Center (Maricopa, AZ) that is periodically outbred with locally caught conspecifics. Insects were reared on green beans and an artificial diet mix (Debolt, 1982) in disposable packs as described (Patana, 1982). Insects were maintained at 25°C under 20% humidity and a L14:D10 photoperiod. *L. lineolaris* were obtained from a laboratory colony maintained at the USDA-ARS Southern Insect Management Research Unit (Stoneville, MS) under conditions similar to those described for *L. hesperus*.

### *Total RNA Extraction, cDNA Synthesis, and Degenerate PCR*

Antennae were harvested from day 5–7 adult male and female *L. hesperus*, frozen immediately and stored at −80°C. Total RNA was isolated from frozen tissues using TRI Reagent RNA Isolation Reagent (Sigma-Aldrich, St. Louis, MO) according to the

manufacturer's instructions. First strand cDNA was synthesized from approximately 1 μg of total RNA using Thermoscript reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamers using conditions recommended by the manufacturer. To identify the *L. hesperus* Orco sequence, degenerate primers (F: 5′ATHAARGCNTGGTAYCCNTGG; R: 5′CYTTYTGRCAYTGYTGRCANAC) were designed to conserved amino acid stretches (IKAWYPW and VCQQCQK) of previously identified Orco sequences. Amplification was performed using 0.7 μL of the cDNA template and 5 μL of each primer with ExTaq DNA polymerase (Takara Bio Inc./Clontech, Madison, WI) and thermocycler conditions consisting of 95°C for 2 min followed by 5 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s, then 5 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, then 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s, and a final 5 min incubation at 72°C. PCR products were electrophoresed on a 1.3% agarose gel and visualized with SYBR Safe (Invitrogen). Amplimers of the expected sizes were gel-excised using an EZNA Gel Extraction kit (Omega Bio-Tek Inc., Norcross, GA), cloned into the pCR4TOPO-TA cloning vector (Invitrogen), and sequenced.

### *Rapid Amplification of cDNA Ends (RACE)*

To obtain the 5′ and 3′ ends of the *L. hesperus* Orco sequence, RACE PCR was performed using cDNAs prepared from 2 μg DNase I-treated antennal RNA with a SMARTer RACE cDNA Amplification kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. Touchdown PCR was performed using ExTaq with 0.5 μL cDNA and the Universal Primer Mix 1 (UPM1) with gene specific primers (LH OR83b F1 RACE—5′ CGCTCTTGACTCGGTCGTACCCAA; LH OR83b R1 RACE—5′ ATCCGTCGTACCAGTGGCAACTGT) and thermocycler conditions consisting of: 95°C for 2 min followed by 5 cycles at 94°C for 30 s, 70°C for 20 s, and 72°C for 90 s, then 5 cycles at 94°C for 30 s, 68°C for 20 s, and 72°C for 90 s, then 30 cycles at 94°C for 30 s, 62°C for 20 s, and 72°C for 90 s, and a final 5 min incubation at 72°C. Nested PCR was then performed with a 0.5 μL aliquot of the first round PCR product using ExTaq and UPM2 with gene specific primers (LH OR83b F2 RACE—5′ GTCGTACCCAACTCCGGCGACCT; LH OR83b F3 RACE—5′ CAGGAGTTGCTGGTCCGCTCTGC; LH OR83b R2 RACE—5′ CAGATGTTTCAGCTGCTCGCAAGC; LH OR83b R3 RACE—5′ GCAGAGCGGACCAGCAACTCCTG) and thermocycler conditions identical to those above. PCR products were electrophoresed on a 1.5% agarose gel and visualized as before. Amplimers of the expected sizes were gel excised, subcloned into the pGEM-T Easy cloning vector (Promega, Madison, WI), and sequenced. Merging the resulting 5′ and 3′ RACE sequence data with the internal fragment identified above gave a full-length cDNA with a single open reading frame (ORF) of the expected size. Gene specific primers designed to encompass the putative start and stop codons (LH OR83b start F—5′ ATGCAGAAAGTGAAGATGCAC and LH OR83b stop R—5′ TTATTTGAGCTGCACCAACAC) were used in multiple independent reactions to amplify the LhOrco ORF. The same primer set was used to amplify the *L. lineolaris* sequence from adult antennae derived cDNAs. Consensus nucleotide sequence data have been deposited with the GenBank database under the accession numbers: LhOrco, JQ639213; and LlOrco, JQ639214.

### *Sequence Analysis*

Comparison of the LhOrco gene sequence with database sequences was performed using BLASTx (http://blast.ncbi.nlm.nih.gov/). Sequences were aligned using the

L-INS-I strategy in MAFFT (Katoh et al., 2005) and rendered with Jalview (Waterhouse et al., 2009). Phylogenetic analysis was performed on the phylgeny.fr server (http://www.phylogeny.fr) (Castresana, 2000; Guindon and Gascuel, 2003; Edgar, 2004; Anisimova and Gascuel, 2006; Chevenet et al., 2006; Dereeper et al., 2010; 2008) using 52 sequences identified from BLAST analysis of the LhOrco sequence. Sequences were aligned with MUSCLE v3.7 and the alignment curated using Gblocks v0.91b with default settings. The phylogenetic tree was constructed using the maximum likelihood method implemented in PhyML v3.0 with default settings and the graphical representation generated using TreeDyn v198.3. Topology and transmembrane domain predictions were performed using TMPred (http://www.ch.embnet.org/software/TMPRED_form.html) (Hofmann and Stoffel, 1993), TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001), Phobius (http://phobius.sbc.su.se/) (Käll et al., 2007), RHYTHM (http://proteinformatics.charite.de/rhythm/) (Rose et al., 2009), TOPCONS (http://topcons.cbr.su.se/) (Bernsel et al., 2009), HMMTOP v2.0 (http://www.enzim.hu/hmmtop/) (Tusnády and Simon, 2001), and TopPred II (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::toppred) (von Heijne, 1992; Claros and von Heijne, 1994).

### *RT-PCR Expression Profile of LhOrco*

Total RNA was extracted using TRI Reagent RNA Isolation Reagent (Sigma-Aldrich) from adult male or female *L. hesperus* antennae (100), legs (150–200), proboscis (50), day 6–9 adult denuded heads (25 heads lacking antennae and proboscis), and day 6–9 adult denuded bodies (3 bodies without heads, legs, or wings). Total RNA was also isolated from the five nymphal developmental stages as well as eggs. First-strand cDNA synthesis was performed using a SuperScript III First-Strand Synthesis System (Invitrogen) with 500 ng DNase I-treated total RNAs and random pentadecamers. A fragment of LhOrco (nt 741–1264) was amplified from the resulting cDNAs using internal primers described above. For control purposes, a fragment of the *L. hesperus* actin ORF (nt 1–554) was amplified using primers (F—ATGTGCGACGAAGAAGTTG; R—GTCACGGCCAGCCAAATC) designed to the *L. lineolaris* sequence (DQ386914). PCR was performed using Sapphire Amp Fast PCR Master Mix (Clontech) with thermocycler conditions consisting of 95°C for 2 min followed by 35 cycles at 94°C for 20 s, 58°C for 20 s, and 72°C for 30 s. Products were electrophoresed on a 1.5% agarose gel and visualized as before.

### *Construction of Insect Expression Plasmids*

Insect expression vectors were constructed to examine the cellular localization and topology of LhOrco. Initially, a fluorescent chimera of LhOrco tagged at either the N or C terminus with enhanced green fluorescent protein (EGFP) was generated via overlap extension-PCR (OE-PCR) (Wurch et al., 1998) using KOD Hot Start DNA polymerase (Toyobo/Novagen, EMD Biosciences, San Diego, CA). N-terminally tagged EGFP-LhOrco was generated using a sense EGFP specific primer (EGFP F—5′ATGGTGAGCAAGGGCG) with a chimeric antisense primer (EGFP-LHOR83b R—5′ GCATCTTCACTTTCTG-CATCTTGTACAGCTCGTCC) and a chimeric sense primer (EGFP-LHOR83b F 5′—GGACGAGCTGTACAAGATGCAGAAAGTGAAGATGC) with the gene specific antisense LH OR83b stop R primer above. C-terminally tagged LhOrco-EGFP was generated using the gene specific sense LH OR83b start F primer above with a chimeric antisense primer (LHOR83b-EGFP R—5′ CTCGCCCTTGCTCACCATTTTGAGCTGCAC-

CAACAC) and a chimeric sense primer (LHOR83b-EGFP F—5′ GTGTTGGTGCAGCT-CAAAATGGTGAGCAAGGGCGAG) with a gene specific antisense EGFP primer (EGFP R—5′TTACTTGTACAGCTCGTCCATG). The initial thermocycler conditions consisted of: 95°C for 2 min followed by 21 cycles at 95°C for 20 s, 58°C for 10 s, and 70°C for 15 s with a final 5 min incubation at 72°C. Thermocycler conditions for joining the respective fragments consisted of: 95°C for 2 min followed by 21 cycles at 95°C for 20 s, 58°C for 10 s, and 70°C for 45 s with a final 5 min incubation at 72°C. The resulting PCR products were gel excised, treated with ExTaq DNA polymerase, cloned into the pIB/V5-His TOPO TA insect expression vector (Invitrogen), and sequence verified. In parallel experiments, PCR primers were used to attach a 6x-His epitope tag to the N terminus of LhOrco. N-terminally tagged LhOrco (His-LhOrco) was generated using a sense primer (His-StuI-LhOR83b F—5′ ATGCATCATCACCATCACCATAGGCCTATGCA-GAAAGTGAAG) containing a new start codon, the 6x-His epitope tag, and a StuI site upstream of the LhOrco sequence and the gene specific antisense LH OR83b stop R primer. As before, PCR was performed using plasmid DNA as a template with KOD Hot Start DNA polymerase and thermocycler conditions consisting of: 95°C for 2 min followed by 21 cycles at 95°C for 20 s, 58°C for 10 s, and 70°C for 30 s with a final 5 min incubation at 72°C. The resulting PCR product was gel excised, treated with ExTaq DNA polymerase, cloned into the pIB/V5-His vector, and sequence verified. C-terminally tagged LhOrco (LhOrco-His) was constructed by subcloning the complete LhOrco ORF minus the stop codon into the pIB/V5-His vector such that the 6x-His epitope tag is derived from the vector sequence; this results in a 45 amino acid C-terminal addition that includes the 6x-His tag. The resulting plasmid was sequence verified as before. For a positive control, a *L. hesperus* Gαi gene product lacking the endogenous stop codon was likewise inserted into the pIB/V5-His expression vector using primers (F—TAATGGGTTGCGCGATCAG; R—GAATAGGCCACAATTTTTTAAGTTT) designed to LhGαi (JF273641).

### *Transient Expression in Cultured Insect Cells*

*Trichoplusia ni* (Tni) cells (Orbigen Inc., San Diego, CA) were maintained as adherent cultures in serum-free insect culture media (Orbigen Inc.). Tni cells seeded into 35 mm #1.5 glass bottom dishes (In Vitro Scientific, Sunnyvale, CA) were transfected with 2 μg plasmid using 4 μL Insect Gene Juice transfection reagent (Novagen, EMD Biosciences, San Diego, CA) for 5 h. Transfection media was then removed, the cells washed twice with 1 mL serum-free media and maintained in serum-free media for 48 h at 28°C. Cells transfected with the EGFP-LhOrco and LhOrco-EGFP chimeras were washed twice with 1 mL IPL-41 insect media (Invitrogen) and imaged in 2 mL IPL-41 using an Olympus FSX-100 fluorescence microscope with FSX-BSW imaging software (Olympus, Center Valley, PA).

### *Immunocytochemistry*

To examine the cellular location of the 6x-His tagged LhOrco constructs, cells 48 h post-transfection were washed twice with 1 mL IPL-41 insect media and then fixed in 3.5% formalin/IPL-41 for 15 min at 4°C. Cells were washed 4× with 1 mL phosphate-buffered saline (PBS) and then blocked for 1 h at 25°C in PBS/10% fetal bovine serum or permeabilized with PBS/10% fetal bovine serum/0.1% Triton X-100. Cells were then incubated for 2 h at 25°C with a rabbit polyclonal anti-His antibody (SC-804, 200 μg/mL; Santa Cruz Biotech. Inc., Santa Cruz, CA) diluted 1:50. After washing, the cells were

incubated for another 2 h at 25°C with a 1:100 dilution of goat anti-rabbit IgG-TRITC (1 mg/mL; SouthernBiotech, Birmingham, AL). Cells were washed again and imaged in 2 mL IPL-41 on an Olympus FSX-100 fluorescence microscope. Images were processed in Adobe Photoshop 7.0 by manually increasing the contrast to +40.

## RESULTS

### *Molecular Cloning and Sequence Analysis of the L. hesperus Orco*

To gain a better understanding of the molecular basis of olfaction in *Lygus*, we sought to clone and characterize *L. hesperus* Orco (LhOrco). Using degenerate primers designed to conserved amino acid stretches of known Orco homologs in conjunction with cDNAs generated from adult *L. hesperus* antennae total RNAs, we obtained an amplimer of the expected fragment size (~760 bp). Sequence analysis revealed that the amplimer was homologous to known Orco sequences. Assembling 5′ and 3′ RACE data generated a 1710 nt cDNA encompassing a 1422 nt ORF with a 249 nt 5′ untranslated region and a 39 nt 3′ UTR/polyA tail. Gene specific primers designed to the putative start and stop codons generated an amplimer of the expected size. The LhOrco ORF encodes a protein of 473 amino acids that has significant sequence identity with Orco proteins from various insect species across a number of orders. Highest sequence identity (63%) was observed with the *Tribolium castaneum* olfactory receptor 16 (CAM84014.1) while 58% identity was observed with *D. melanogaster* OR83b (AAT71306.1), the first Orco identified. Given the functional importance of Orco in mediating insect olfaction, we sought to determine the degree of sequence conservation in the closely related species, *L. lineolaris*. Using the same primer set used to clone the LhOrco ORF, we generated an amplimer of identical length from adult *L. lineolaris* antennae cDNAs. Sequence comparison between the LhOrco and LlOrco coding sequences revealed that they were 100% identical (Fig. 1), supporting the essential function this gene product has in insect olfaction.

An atypical membrane topology (intracellular N terminus and extracellular C terminus) has been demonstrated for both the *D. melanogaster* Orco (DmOrco) and *Anopheles gambiae* Orco (AgOrco) (Benton et al., 2006; Lundin et al., 2007; Tsitoura et al., 2010). We consequently sought to computationally determine the membrane topology of LhOrco. TMHMM2.0 has been frequently used to predict the membrane topology and TM location of Orco orthologs (Krieger et al., 2003; Lu et al., 2009; Wang et al., 2012); however, the program predicted 6TMs for LhOrco with both termini intracellular (Fig. 2). To reconfirm the utility of the prediction algorithm, the DmOrco and the putative *Sitobion avenae* Orco (ACT37280) sequences were likewise analyzed. Both were predicted to contain 7TMs with the expected atypical membrane topology (data not shown) suggesting that there may be some unique feature to the LhOrco sequence. We therefore sought to obtain a consensus across several prediction algorithms. HMMTOP, RHYTHM, and TOPCONS, the latter of which provides a consensus prediction based on five other programs, all predicted 6TMs with intracellular N and C terminal tails (Fig. 2). Phobius predicted an intracellular N terminus and an extracellular C terminus but found only 5TMs, whereas TMpred and TopPred II predicted 7TMs and the characteristic atypical membrane topology.

Aligning the LhOrco amino acid sequence with Orco sequences from insect species representing six different insect orders (Diptera, Lepidoptera, Coleoptera, Hemiptera, Hymenoptera, and Orthoptera), revealed significant sequence conservation and suggested the likely presence of 7TMs (Fig. 3). Indeed, residues that comprise portions of

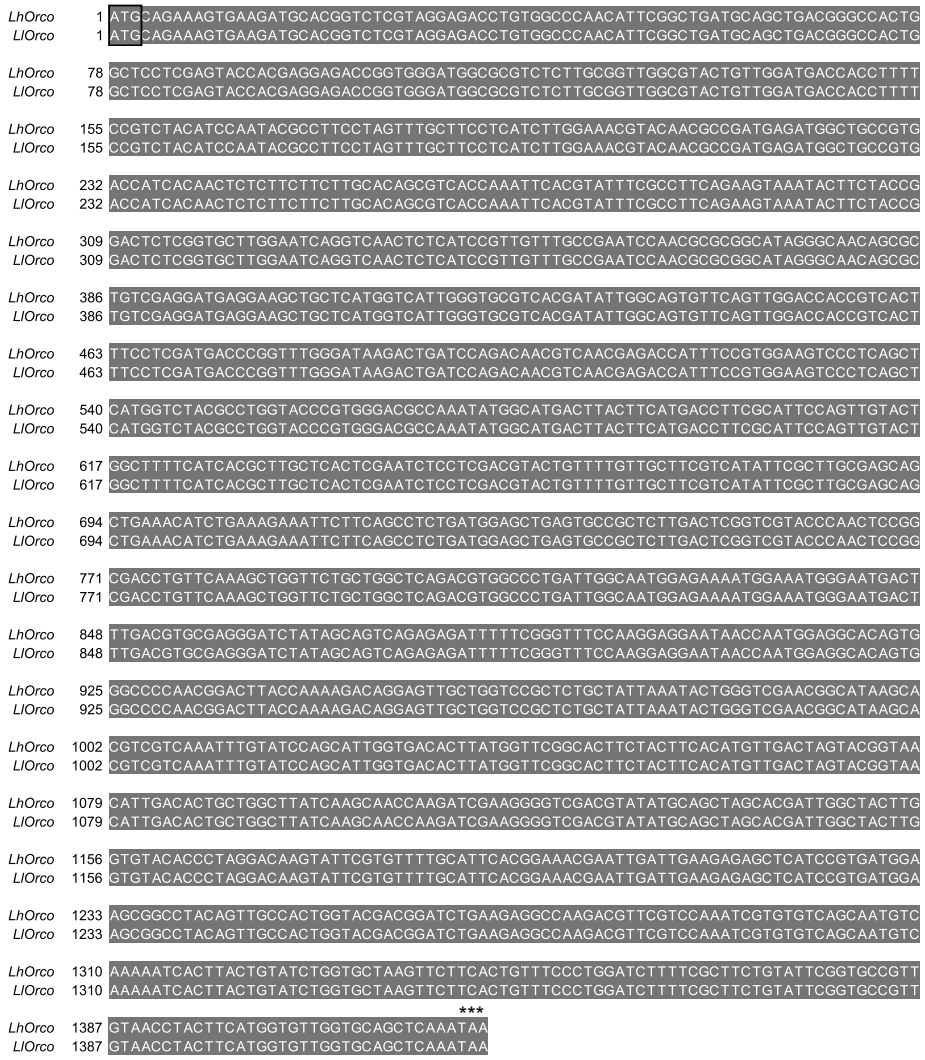


**Figure 1.** Nucleotide alignment of the *Lygus hesperus* and *Lygus lineolaris* olfactory co-receptor (Orco) ORFs. The start codon (ATG) is shown boxed and the stop codon is indicated by ***.

TMs 6 and 7 are reasonably conserved in LhOrco in terms of position and side-chain character. More specifically, residues 393–399 (TVVGYLG) in DmOrco TM6 are thought to comprise an ion selectivity motif with Val395 and Leu398 playing critical roles (Wicher et al., 2008). This motif is moderately conserved in LhOrco (residues 380–386; STIGYLV) with the Leu398 position absolutely conserved and the hydrophobic character of the Val395 position conserved (Ile vs. Val); only the GYL portion of the ion selectivity motif appears to be absolutely conserved across species. In addition, a Tyr residue (Y464) located in *B. mori* Orco TM7 that was recently identified as essential for ion channel function (Nakagawa et al., 2012) is likewise conserved in LhOrco (Y465) (Fig. 3). Significant sequence conservation was also observed in ICL3 (i.e., residues 398–446), a region that is thought to mediate Orco interactions with conventional ORs (Benton et al., 2006).

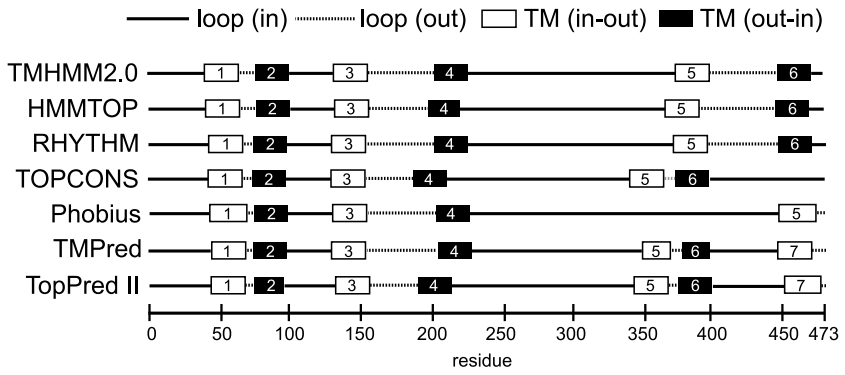


**Figure 2.** Predicted topology and transmembrane domain location of LhOrco. Topology and transmembrane domain predictions based on the LhOrco coding sequence were performed with TMHMM v2.0 (Krogh et al., 2001), HMMTOP v2.0 (Tusnády and Simon, 2001), RHYTHM (Rose et al., 2009), TOPCONS (Bernsel et al., 2009), Phobius (Käll et al., 2007), TMPred (Hofmann and Stoffel, 1993), and TopPred II (von Heijne, 1992; Claros and von Heijne, 1994). Portions of the sequence predicted to be intracellular are indicated by solid lines and extracellular by dotted lines. Numbered TM domains are indicated by boxes with those predicted to exhibit an in–out orientation shown in white and those with an out–in orientation shown in black.

Similar to other Orco sequences, the extended LhOrco ICL2 (117 amino acids comprising residues 217–333 is reasonably well conserved, which is consistent with a potential role in coupling Orco to intracellular transport mechanisms (Benton et al., 2006).

Phylogenetic analysis of the LhOrco sequence using a neighbor-joining tree (maximum likelihood method implemented in PhyML) was consistent with the inferred phylogeny of the species analyzed with the holometabolous coleopteran, hymenopteran, dipteran, and lepidopteran Orcos clustering together. In contrast, both *Lygus* sequences clustered as expected with the hemimetabolous insects including other members of the hemipteran assemblage (i.e., aphids, lice, and bugs) and orthopterans (Fig. 4).

### *RT-PCR Expression Profile of LhOrco*

Orco transcripts are frequently expressed in multiple olfactory tissues. We consequently performed nonquantitative RT-PCR to examine the spatial, sex specific, and temporal expression of LhOrco. Sequence specific primers were designed to amplify either a 524 bp fragment of LhOrco or a 555 bp fragment of the control gene (actin). In adult males, LhOrco expression was highest in antennae (Fig. 5A). Less robust expression was observed in adult male legs, proboscis, and bodies trimmed of legs, heads, and wings. No amplimer was observed in adult male heads trimmed of antennae. Sequence analysis of a second, higher molecular weight (703 bp) amplimer observed in male bodies, and to a lesser degree in male legs, indicated that it was the product of nonspecific amplification. In adult females, LhOrco expression was likewise highest in antennae (Fig. 5A). Lower levels of expression were observed in female legs and bodies and LhOrco expression in female proboscis was barely detectable. A faint but distinct amplimer was detected in female heads (Fig. 5A). LhOrco expression was not restricted to adults as amplimers of the expected size were clearly detected in eggs and throughout nymphal development (Fig. 5B). As a measure of cDNA integrity, the cytoplasmic actin gene was amplified in tandem with LhOrco. In all cases a robust product of the expected size was observed.

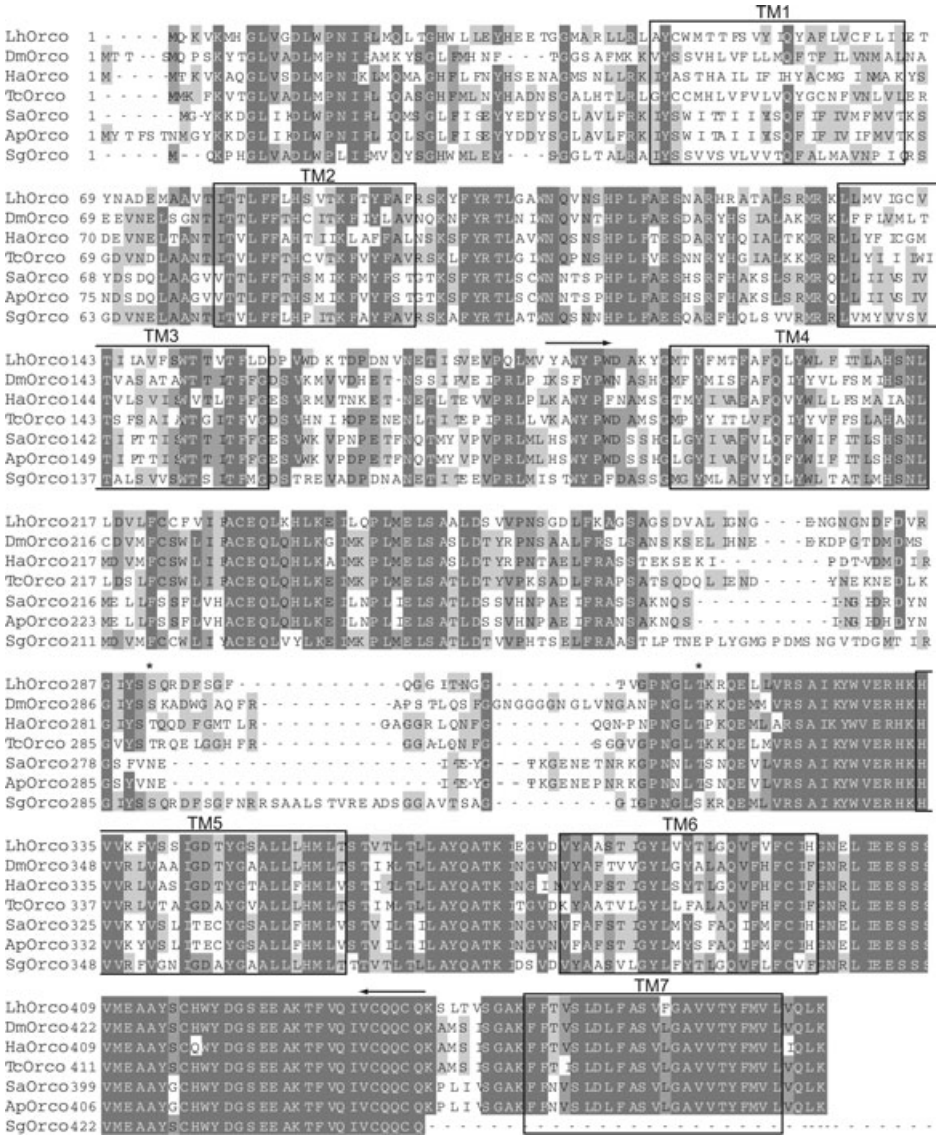


**Figure 3.** Amino acid sequence alignment of LhOrco with representative Orco orthologs from six insect orders. Sequences were aligned using the L-INS-I strategy in MAFFT (Katoh et al., 2005) and rendered with Jalview (Waterhouse et al., 2009). Numbers to the left indicate the position of the first residue in each line. Abbreviations—Lh, *Lygus hesperus* (JQ639213—Hemiptera); Dm, *Drosophila melanogaster* (NP524235—Diptera); Ha, *Helicoverpa armigera* (ADQ13177—Lepidoptera); Tc, *Tribolium castaneum* (CAM84014—Coleoptera), Sa, *Sitobion avenae* (ACT37280—Hemiptera); Ap, *Apis mellifera* (NP001128415—Hymenoptera); and Sg, *Schistocerca gregaria* (AEX28371—Orthoptera). The positions of the putative transmembrane domains (TM1–7) are indicated in relation to the DmOrco sequence. Arrows indicate the position and orientation of the degenerate primers used in cloning LhOrco; asterisks (*) indicate the location of putative protein kinase C sites (Ser291 and Thr314) in ICL2 that may be involved in the regulation of Orco function.

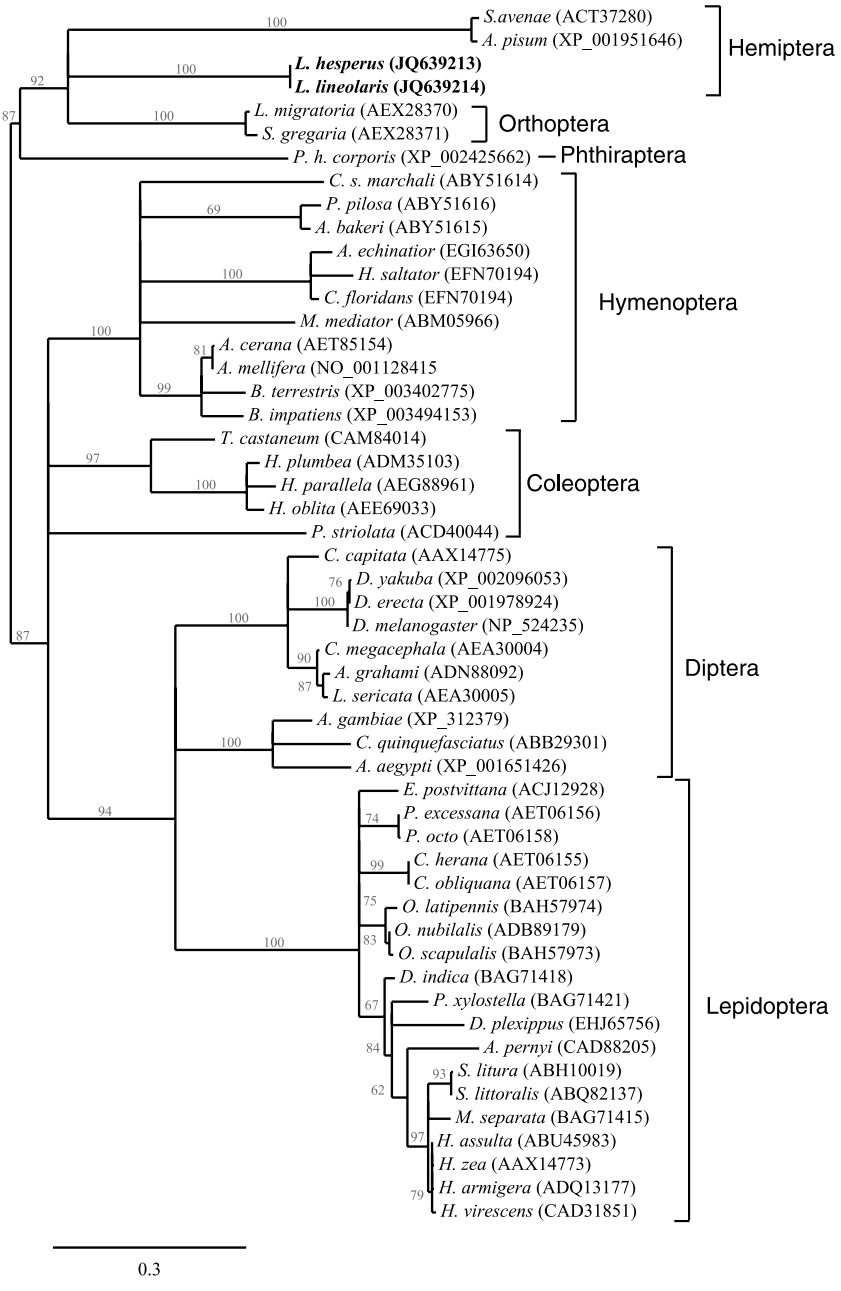


**Figure 4.** Neighbor-joining tree of Orco orthologs from various insect species. Phylogenetic analysis was performed using 52 sequences identified from BLAST analysis of the LhOrco sequence. Sequences were aligned with MUSCLE v3.7 and the alignment curated using Gblocks with default settings. The phylogenetic tree was constructed using the maximum likelihood method implemented in PhyML v3.0 with default settings and the graphical representation generated using TreeDyn. Bootstrap support values above 65% are shown. Accession numbers are indicated in parenthesis to the right of each species with the representative insect order clustering indicated. The LhOrco and LlOrco sequences are shown in bold.

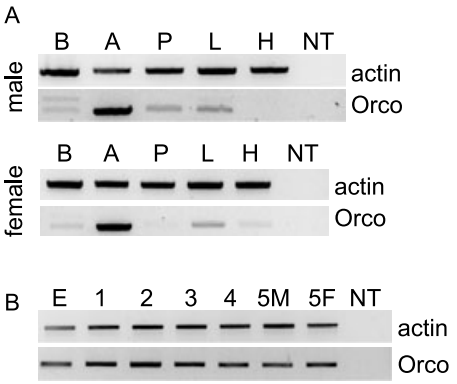


**Figure 5.** RT-PCR transcript expression profile of LhOrco. (A) Expression in adult male and female tissues. cDNAs prepared from: day 6–9 bodies lacking heads, legs, and wings (B); antennae (A); proboscis (P); legs (L); day 6–9 heads lacking antennae and proboscis (H), no template (NT). (B) Developmental expression profile. cDNAs prepared from: eggs (E); first through fourth instar nymphs (1–4); fifth instar male nymphs (5M), fifth instar female nymphs (5F), no template (NT). The *Lygus* actin gene was amplified from each tissue as an internal control. Products were analyzed on 1.5% agarose gels and stained with SYBR Safe.

### *Subcellular Localization and Membrane Topology of LhOrco*

Despite significant sequence identity with known Orco proteins, the consensus membrane topology predicted for LhOrco was for both the N and C termini to be intracellular (Fig. 2). We consequently sought to examine the validity of these prediction algorithms using immunofluorescent microscopy with differentially tagged LhOrco proteins transiently expressed in cultured insect cells. A similar methodology was previously used to experimentally confirm the atypical topologies of DmORs and AgOrco (Smart et al., 2008; Tsitoura et al., 2010). To facilitate confirmation of expression and to provide insights into LhOrco intracellular trafficking, we initially tried to examine the localization of LhOrco chimeras tagged at either the N or C terminus with EGFP. Each construct was transiently expressed in cultured Tni cells and the fluorescence expression profile examined 48 h after transfection. Both N-terminally tagged EGFP-LhOrco and C-terminally tagged LhOrco-EGFP exhibited a diffuse cytosolic fluorescence profile reminiscent of that described (Thomas et al., 1998) for endoplasmic reticulum localization (Fig. 6A). Neither construct exhibited the cell surface expression profile expected, suggesting that normal trafficking of the receptor had been impaired by addition of the bulky EGFP tag. To eliminate these adverse localization affects, we replaced the EGFP tag with a 6x-His tag. N-terminally tagged LhOrco (His-LhOrco) was generated by OE-PCR while C-terminally tagged LhOrco (LhOrco-His) was generated by removing the endogenous stop codon from the LhOrco sequence, thereby allowing introduction of the plasmid-derived 6x-His tag. Prior to GPCR activation, the Gα subunits of heterotrimeric G proteins are frequently localized to the intracellular surface of the plasma membrane (Marrari et al., 2007). We consequently generated a *L. hesperus* Gαi (JF273641) construct incorporating a C terminal 6x-His tag for use as a positive control. Immunofluorescence analyses were performed in Tni cells 48 h after transfection using a polyclonal anti-His antibody in conjunction with a TRITC-tagged anti-rabbit antibody. In the absence of cell permeabilization, plasma membrane-associated fluorescence was only observed in cells expressing LhOrco-His (Fig. 6B). No fluorescence was observed in nontransfected cells, cells transfected with the control His-Gαi protein, or cells transfected with His-LhOrco. In contrast,

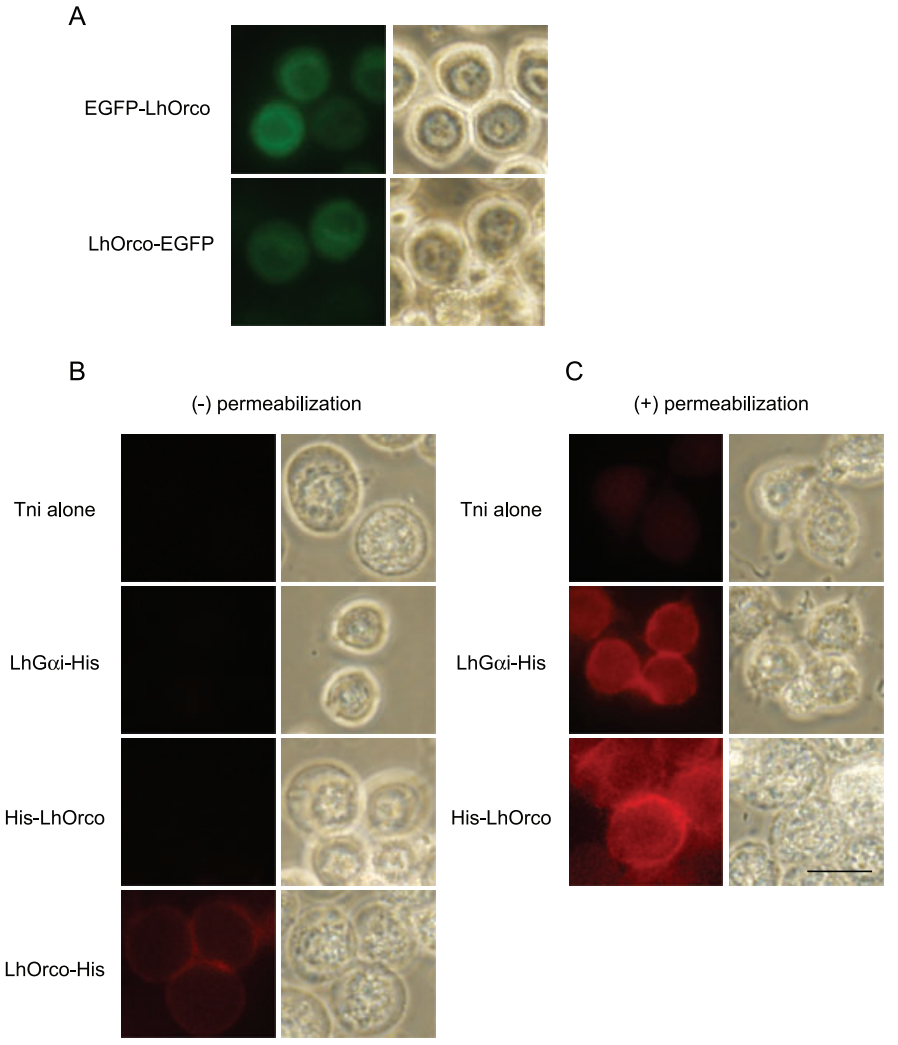


**Figure 6.** Localization and topological orientation of transiently expressed LhOrco in cultured Tni cells. (A) Expression of LhOrco-EGFP fluorescent chimeras. Chimeras were generated by OE-PCR with EGFP at the N terminus (EGFP-LhOrco) or C terminus (LhOrco-EGFP). No cell surface localization was observed in either construct. (B) Fluorescent immunohistochemistry of 6x-His tagged LhOrco in the absence of cell permeabilization. Fixed Tni cells transfected with plasmids encoding LhGαi-His (C-terminal 6x-His tag), His-LhOrco (N-terminal 6x-His tag), LhOrco-His (C-terminal 6x-His tag), or mock transfected (Tni alone) were probed with a polyclonal mouse anti-His antibody (primary) and a goat anti-mouse IgG-TRITC antibody (secondary). In the absence of cell permeabilization, fluorescence was only observed in cells expressing LhOrco-His, indicating an extracellular C terminus. (C) Fluorescent immunohistochemistry of 6x-His tagged LhOrco following cell permeabilization. Cells are as in (B). In the presence of cell permeabilization, fluorescence is seen in the His-LhOrco cells, indicating an intracellular N terminus. Fluorescence, as expected, was also observed in the LhGαi-His expressing cells. Scale bar represents 20 μm. Images are representative of multiple independent transfections.

plasma membrane-associated fluorescence was observed in both His-Gαi transfected cells and His-LhOrco cells following permeabilization with 0.1% Triton-X100 (Fig. 6C). Taken together, these results demonstrate that the LhOrco C terminus is extracellular while the N terminus is intracellular. LhOrco thus adopts the atypical membrane topology similar

to that reported for other Orco proteins, a topology that was predicted by only three of seven prediction algorithms.

## DISCUSSION

In this study, we cloned and characterized Orco orthologs from two agronomically important pest species, the western tarnished plant bug (*L. hesperus*) and its sympatric sister species the tarnished plant bug (*L. lineolaris*). Despite millions of years of evolutionary separation, the two *Lygus* Orco sequences exhibit significant conservation with Orco orthologs identified in other insect species (Krieger et al., 2003; Melo et al., 2004; Pitts et al., 2004; Jones et al., 2005; Smadja et al., 2009; Kirkness et al., 2010; Wang et al., 2012; Yang et al., 2012). This conservation has been shown to extend beyond primary sequence to include functionality as diverse Orco genes were able to functionally complement Orco-deficient *D. melanogaster* (Jones et al., 2005). The heteromeric complexes formed following Orco interactions with ligand-specific ORs are ligand-gated ion channels that open in response to odorants (Nichols et al., 2011; Pask et al., 2011; Nakagawa et al., 2012). Consistent with this mechanistic view of odorant perception, LhOrco exhibits significant sequence conservation (Fig. 3) across ICL3 and portions of TMs 6–7, regions of Orco that have been shown to be important for forming interactions with ligand-specific ORs and ion pore functionality (Benton et al., 2006; Wicher et al., 2008; Nakagawa et al., 2012). In addition, DmOrco has recently been shown to be regulated by protein kinase C dependent phosphorylation (Sargsyan et al., 2011). Two of the potentially phosphorylated sites in DmOrco are conserved in LhOrco ICL2 (Ser291 and Thr314).

Despite exhibiting the 7TM domain feature characteristic of GPCRs, Orco differs from GPCRs in that it has an inverted membrane topology, a feature that has been experimentally confirmed for both DmOrco and AgOrco (Benton et al., 2006; Lundin et al., 2007; Tsitoura et al., 2010). The atypical membrane topology has also been predicted for Orco orthologs from a number of insect species with TMHMM2.0 and HMMTOP among the most frequently cited prediction algorithms (Benton et al., 2006; Lundin et al., 2007; Malpel et al., 2008; Lu et al., 2009; Wang et al., 2012). The two algorithms, however, predicted LhOrco to exhibit a membrane topology with both termini intracellular (Fig. 2). Similar results were obtained using a number of other algorithms with two of five additional programs predicting the N-in C-in topology. In vitro experiments designed to probe the membrane topology of LhOrco with an antibody under permeabilized and non-permeabilized conditions invalidated those predictions and demonstrated that LhOrco assumes the atypical inverted membrane topology characteristic of Orco proteins (Fig. 6B and C). Consequently, our study suggests that TMpred and TopPred II are the best-adapted algorithms for predicting Orco TM topology. While Phobius was the only other program to accurately predict LhOrco topology, it was less robust at predicting TM location and number.

Previous studies examining Orco topology were conducted in cultured insect cells; *Drosophila* S2 cells with DmOrco (Benton et al., 2006; Lundin et al., 2007) and lepidopteran-derived cell lines (Bm5 and High Five) with AgOrco (Tsitoura et al., 2010). Another lepidopteran cell line, Sf9, was employed to examine the topology of *Drosophila* ligand-specific ORs (Smart et al., 2008). The latter two studies demonstrated the utility of lepidopteran cell lines for heterologous expression of dipteran ORs/Orco. In the current study, we expanded on that utility by using cultured Tni cells (a cell line derived from cabbage looper ovarian cells) to determine the membrane topology of LhOrco.

As discussed above, His-tagged LhOrco was expressed at the cell surface as expected. The cell line, however, proved inadequate for expressing LhOrco-EGFP chimeras (Fig. 6A). Fluorescent proteins are frequently used to facilitate the localization of cell surface receptors with minimal affects on trafficking (Giepmans et al., 2006). Indeed, Benton et al. (2006) demonstrated cell surface expression of DmOrco fluorescent chimeras in both salivary gland cells and olfactory sensory neurons. We therefore suspect that the observed impaired trafficking of the LhOrco-EGFP chimeras either resulted from protein misfolding due to occlusion of protein stabilization sites or that a critical plasma membrane targeting sequence was hidden. In both cases, our data suggest that the Tni cell line may lack certain components of the intracellular transport machinery endogenous to salivary gland cells and olfactory sensory neurons that facilitated the translocation of fluorescent Orco chimeras.

Consistent with a role in olfaction, LhOrco transcripts were predominantly localized to the chemosensory organs (i.e., antennae, proboscis, and legs) with highest expression observed in adult antennae. Even though sexual dimorphism in antennae length and antennal sensilla number has been reported in *L. lineolaris* (Chinta et al., 1997) no sex-based differences in LhOrco antennae expression were observed (Fig. 5A). Male dominant LhOrco expression, however, was observed in the adult proboscis. While the proboscis is generally associated with gustatory perception, the expression of Orco in this chemosensory organ is not without precedence (Krieger et al., 2003; Melo et al., 2004; Pitts et al., 2004; Xia and Zwiebel, 2006; Malpel et al., 2008; Wang et al., 2012) and may reflect an olfactory function similar to that described for the olfactory neurons in the *A. gambiae* proboscis that respond to human-derived odors (Kwon et al., 2006). Potentially in support of this role, the labium tip of *L. rugulipennis* (European tarnished plant bug) has been shown to be important for detecting cues regarding plant health prior to oviposition (Conti et al., 2011). LhOrco expression in the proboscis and legs could also be indicative of a role in contact chemosensory perception. A subset of *Drosophila* sensory neurons that co-express Orco along with ORs and gustatory receptors has been identified (Fishilevich and Vosshall, 2005) and Orco transcripts have been amplified from the legs of a wide number of species including locusts (Yang et al., 2012), fig wasps (Lu et al., 2009), mosquitoes (Melo et al., 2004; Pitts et al., 2004; Xia and Zwiebel, 2006), and the blowfly (Wang et al., 2012). The presence of LhOrco transcripts in eggs (Fig. 5B) is likewise in agreement with previous findings as Orco orthologs have been detected in blowfly eggs (Wang et al., 2012) and embryonic stages of the fruitfly and yellow fever mosquito (Larsson et al., 2004; Melo et al., 2004). The continuous expression of LhOrco throughout the nymphal stages is also consistent with that reported for other hemimetabolous insects (Yang et al., 2012). Orco orthologs in holometabolous insects, in contrast, appear to undergo a significant decline during the pupal stage (Melo et al., 2004; Wang et al., 2012)

Based on similarities in sequence, membrane topology and expression profile, we feel that it is reasonable to conclude that LhOrco, similar to that of Orco orthologs in other insects, plays a critical role in mediating odorant perception and discrimination. Consequently, targeted disruption of this functionality could present a novel means of controlling pest populations (Jones et al., 2005). In support of this approach, *Drosophila* expressing Orco null mutants were reported to exhibit severely impaired responses to behaviorally active single odors (Larsson et al., 2004). In addition, RNA interference-mediated knockdown of Orco orthologs in a parasitic wasp (*Microplitis mediator*) and a striped flea beetle (*Phyllotreta striolata*) affected Y-tube olfactometer responses (Li et al., 2012) and behavioral discrimination of attractant and repellent stimuli (Zhao et al., 2010). Disruption of normal Orco function has also been achieved with a small molecule agonist

that triggers the opening of Orco-based ion channels, an effect that could potentially overwhelm the insect sensory system and thus make odor differentiation difficult (Jones et al., 2011). Given the relative success of these approaches, it is likely that similar methods may be used to facilitate the development of novel control strategies that specifically target *Lygus* spp. populations in the field. Further studies of LhOrco are also expected to significantly advance not only our molecular understanding of olfaction within *Lygus* species, which is currently limited to a single odorant binding protein, but also our understanding of hemipteran olfactory systems.

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