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Molecular characterization and immunolocalization of the olfactory co-receptor Orco from two blood-feeding muscid flies, the stable fly (*Stomoxys calcitrans*, L.) and the horn fly (*Haematobia irritans irritans*, L.)

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Abstract

Biting flies are economically important blood-feeding pests of medical and veterinary significance. Chemosensory-based biting fly behaviours, such as host/nutrient source localization and ovipositional site selection, are intriguing targets for the development of supplemental control strategies. In an effort to expand our understanding of biting fly chemosensory pathways, transcripts encoding the highly conserved insect odorant co-receptor (Orco) were isolated from two representative biting fly species, the stable fly (*Scal\Orco*) and the horn fly (*Hirr\Orco*). Orco forms a complex with an odour-specific odorant receptor to form an odour-gated ion channel. The biting fly transcripts were predicted to encode proteins with 87–94% amino acid similarity to published insect Orco sequences and were detected in various immature stages as well as in adult structures associated with olfaction, i.e. the antennae and maxillary palps, and gustation, i.e. the proboscis. Further, the relevant proteins were immunolocalized to specific antennal sensilla using anti-serum raised against a peptide sequence conserved between the two fly species. Results from the present study provide a basis for functional evaluation of repellent/attractant effects on as yet uncharacterized stable fly and horn fly conventional odorant receptors.

Keywords: Orco, biting flies, olfaction.

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Introduction

Stable flies, *Stomoxys calcitrans* L., and horn flies, *Haematobia irritans* L. are significant livestock pests that are problematic in confined (Campbell *et al*., 1987; Wieman *et al*., 1992) and rangeland operations (Kunz *et al*., 1984; Byford *et al*., 1992; Campbell *et al*., 2001; Broce *et al*., 2005). Their economic impact on the US cattle industry is astounding, having a $1–2.2 billion impact on cattle production annually (Cupp *et al*., 1998; Taylor *et al*., 2012). Both biting fly species primarily parasitize livestock, yet exhibit different associations with their host and use different substrates for development of the immature stages. The horn fly spends the majority of its time somewhat permanently associated with its bovine host, feeding 10–40 times per day and leaving only periodically to oviposit in fresh manure pats or to migrate short distances (Campbell, 2006). The stable fly tends to perch on resting sites where it 'ranges' for a host (Gibson & Torr, 1999), takes 1–2 bloodmeals per day, and spends in comparison less time on-animal (Foil & Hogsette, 1994). The stable fly is also a much more cosmopolitan pest with evidence of feeding on humans (Newson, 1977; Koehler & Kaufman, 2006), dogs (Pitzer *et al*., 2011), and pelicans (Johnson *et al*., 2010) and of using sugar as an alternative energy resource (Jones *et al*., 1985, 1992; Taylor & Berkebile, 2008). Immature horn fly development requires fresh cattle manure, as it provides an optimum moisture level and bacterial community on which immature development can succeed (Kuramochi, 2000; Perotti *et al*., 2001); stable fly larval habitats are more varied, comprised of decaying organic matter, such as spilled hay, mixed with moisture from host urine and manure or rain that enables persistence of a microbe-rich community (Romero *et al*., 2006; Talley *et al*., 2009). Sugar cane debris (Koller *et al*., 2009) and biosolid cakes at wastewater treatment facilities (Doud *et al*., 2012) are also substrates in which immature stable fly development has been observed.

Olfaction plays a critical role in biting fly host localization (Gibson & Torr, 1999; Birkett *et al*., 2004; Jeanbourquin & Guerin, 2007b; Oyarzún *et al*., 2009) and ovipositional site selection (Perotti *et al*., 2001; Romero *et al*., 2006; Jeanbourquin & Guerin, 2007a; Tangtrakulwanich *et al*., 2011), and the biological/behavioural differences between the stable fly and the horn fly may result in their displaying a varied repertoire of chemosensory gene products. Insect olfaction has been studied extensively in several model dipteran and lepidopteran species (Jacquin-Joly & Merlin, 2004; Dahanukar *et al*., 2005; Pelosi *et al*., 2006). The conversion of a volatile to a nervous impulse directed to the insect brain centre begins with porous sensilla (hairs) associated with the cuticle of the olfactory organs, i.e. the antennae and maxillary palps. Each sensillum houses between one and four specialized olfactory sensory neurons (OSNs), the dendrites of which extend into the sensillar lymph. Upon exposure to an odour plume, odorant-binding proteins present in the sensillar lymph are believed to bind a hydrophobic volatile and shuttle it to ligand/odorant-selective odorant receptors (ORs) bound to dendritic membranes. An individual OSN typically expresses a single OR (one neuron, one receptor; Couto *et al*., 2005) although there are known exceptions to this relationship (Fishilevich & Vosshall, 2005; Goldman *et al*., 2005). Once the ligand/odorant is bound, a cascade of events is initiated that leads to nervous activity, but the mechanism by which this occurs remains unclear. An elegant set of studies by Sato *et al*. (2008) and Wicher *et al*. (2008) redefined our understanding of insect ORs as ligand-gated ion channels. A functional channel is comprised of an OR that heterodimerizes with Orco, an odorant co-receptor that is highly conserved in Insecta (Krieger *et al*., 2003) and is absolutely critical for OR stability (Benton *et al*., 2006). A newly described class of chemosensory receptors, members of the ionotropic receptor family, has recently been described in *Drosophila* (Benton *et al*., 2006). Interestingly, the receptors are expressed in a subset of OSNs that are distinct from OSNs expressing OR-Orco (Benton *et al*., 2009), underscoring the complexity of insect odour recognition pathways.

Manipulation of insect olfaction for the development of control technologies based on insect behaviour modification (Bohbot & Dickens, 2012) would be useful in an integrated pest management programme and is desirable for biting fly pests, especially given the increasing development of insecticide resistance in horn flies (reviewed by Oyarzún *et al*., 2008) and the low level of adult stable fly control achieved using insecticides owing to the minimal time they spend feeding on their host. Examples of such manipulation include modulation of the OR-Orco complex, one mechanism of action for the insect repellent DEET (Ditzen *et al*., 2008; Pellegrino *et al*., 2011), and activation or inhibition of Orco (Chen & Luetje, 2012; Jones *et al*., 2012). The biochemical pathway regulating chemosensation in the stable fly and the horn fly warrants further study to improve our understanding of the biting fly response to attractants/repellents and their utility for enhancing pest control. While Olafson *et al*. (2010) identified a number of genes with a putative role in stable fly chemosensation, including two unique ORs, no published reports are available for such genes in the horn fly. In the present study, *Orco* was isolated from the stable fly and the horn fly and its temporal and spatial expression pattern as well as its localization to OSNs within antennal sensilla are described.

Results and discussion

Isolation of Orco orthologues from Muscidae

Degenerate-primed PCR was used to isolate 660-bp and 672-bp fragments from the stable fly and the horn fly, respectively, which demonstrated 89% nucleotide identity to each other and 82–83% nucleotide sequence identity to *Orco* from two calliphorid species, *Chrysomya megacephala* (F.) and *Chrysomya rufifacies* (Macquart). These fragments enabled isolation of full-length *Orco* cDNA sequences from the stable fly and horn fly that encoded 478 aa and 485 aa proteins, respectively, both of which are predicted to contain seven transmembrane (TM) domains, a signature of OR sequences (Fig. 1A). Further, the coding sequences displayed 87–94% amino acid identity to Orco from *Drosophila melanogaster* Meigen, *Bactrocera curcurbitae* Coquillet and *C. rufifacies*, and phylogenetic comparison of dipteran Orco revealed that the muscid sequences shared a common node with Orco from calliphorid fly species (Fig. 1B). As a result, the muscid transcripts were named *Scal\Orco* and *Hirr\Orco*, and the corresponding predicted amino acid sequences were designated ScalOrco and HirrOrco, respectively. Analysis of membrane topology using the TopPred, TMHMM, and TMPred algorithms indicated that the biting fly Orco sequences have an intracellular N-terminus and an extracellular C-terminus, an organization similar to that which has been experimentally confirmed for Drosophila ORs (Benton *et al*., 2006; Lundin *et al*., 2007). HirrOrco is seven amino acids (^{254}SAALLPN^{260}) longer than ScalOrco and these residues are located in the predicted intracellular loop connecting TM4 and TM5 (Fig. 1A), a region thought to be important for intracellular transport (Benton *et al*., 2006). Conservation of residues within the C-terminus (predicted TM6-TM7) has been observed for conventional *Drosophila* ORs and insect Orco sequences, and Benton *et al*. (2006) demonstrated that the loop connecting TM6 and TM7 is part of a region where OR and Orco interact. The predicted TM6 and TM7 of both ScalOrco and HirrOrco display a high level of sequence conservation with other insect Orcos, including the tyrosine

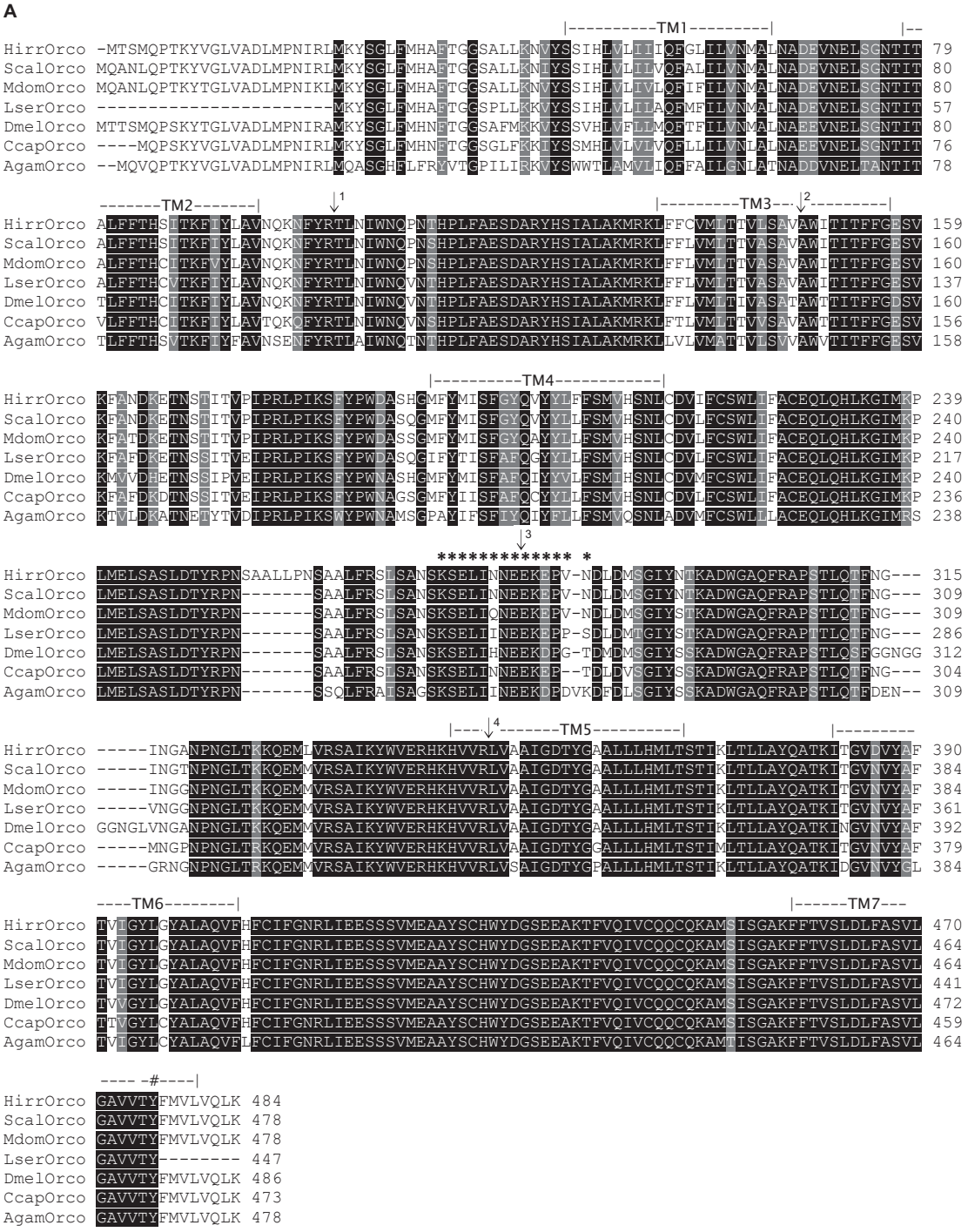

Figure 1. Sequence conservation of ScalOrco and HirrOrco. (A) Amino acid sequence alignment of ScalOrco and HirrOrco to protein sequences from related dipterans. Identical residues are shaded, and the predicted transmembrane domains TM1–TM7 are identified by dashed bars. Location of four known introns in the partial *Scal\Orco* genomic sequence are identified as ↓1–4; the location of intron 1 (↓1) is shared by the horn fly. Asterisks indicate the 14 amino acid peptide used to generate polyclonal antibodies and # indicates the conserved tyrosine (Y) residue in TM7 (Y478 in *Drosphila melanogaster*). (B) Phylogenetic analysis of dipteran Orco sequences. The neighbour-joining tree was constructed with Orco sequences from the indicated dipteran species, and bootstrap values are based on 1000 replicates with support values >80% displayed on nodes of the tree. Abbreviations and relevant accession numbers are as follows: Aaeg (*Aedes aegypti*, EAT42706); Agam (*Anopheles gambiae*, XP_312379); Agra (*Aldrichina graham*, ADN88092); Bcuc (*Bactrocera curcurbitae*, ADK97803); Bdor (*Bactrocera dorsalis*, ACC86853); Ccap (*Ceratitis capitata*, AAX14775); Cmeg (*Chrysomya megacephala*, AEA30004); Cqui (*Culex quinquefasciatus*; ABB29301); Dana (*Drosophila ananassae*, XP_001953343); Dmel (*D. melanogaster*, AAT71306); Dyak (*Drosophila yakuba*, XP_002096053); Hirr (*Haematobia irritans*, ACF21678); Lser (*Lucilia sericata*, AEA30005); Mdom (*Musca domestica*, AFH96944); Scal (*Stomoxys calcitrans*; ACF21677).

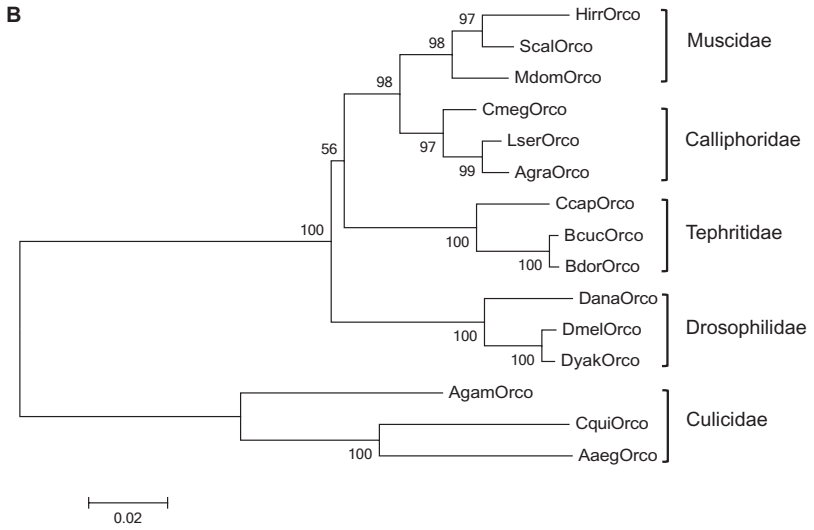

Figure 1. *Continued*

residue in TM7 (Y478 in *D. melanogaster*) that is important for successful OR-Orco interactions *in vivo* (Nakagawa *et al.*, 2012). Sequence conservation within the C-terminal region of ScalOrco and HirrOrco may translate to functional conservation, suggesting that they may be able to dimerize with ligand/odorant-selective ORs.

Immunolocalization of ScalOrco and HirrOrco to antennal sensilla

Stable fly and horn fly antennae comprise a scape, pedicel and flagellum, and antennal sensilla are predominantly located in the flagellum (Figs 2A and 3A; Lewis, 1971; White & Bay, 1980; Tangtrakulwanich *et al.*, 2011). In order to localize the expression of Orco, affinity-purified polyclonal antibodies were generated against a 14 amino acid synthetic peptide conserved between ScalOrco and HirrOrco (Fig. 1A), and the antibody was used in immunohistochemical evaluation of head cryosections containing antennae from the stable fly and horn fly. Antibody binding was detected in numerous distinct cell bodies within the respective flagella, as well as in the dendritic extension of a number of OSNs that project into antennal sensilla (Figs 2C and 3C), supporting Orco expression in the majority of antennal OSNs. It was difficult to identify an optical plane that could be used to trace the labelling from the cell

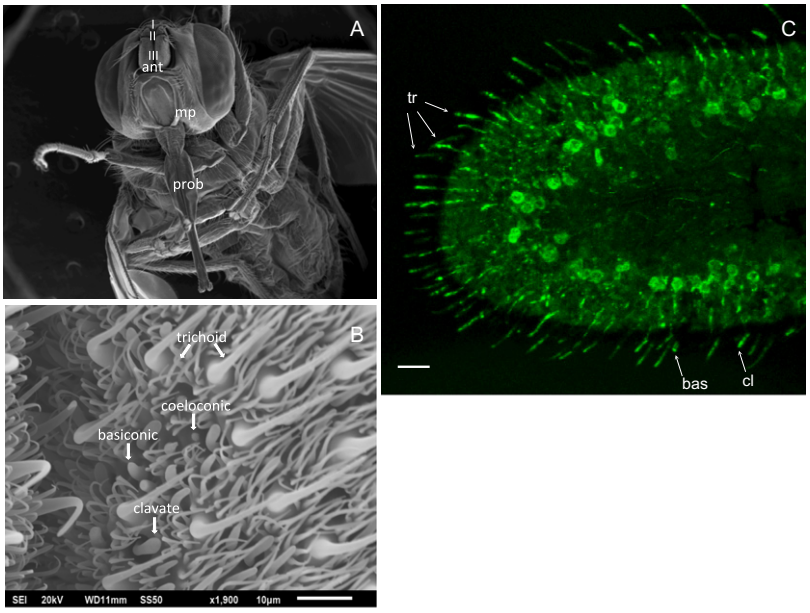

Figure 2. Localization of ScalOrco in *Stomoxys calcitrans* female antenna. Green fluorescence represents anti-Orco detected using an Alexa Fluor 488-conjugated secondary antibody. (A) Scanning electron microscopy of a stable fly head depicting the three antennal (ant) segments (I: scape, II: pedicel, III: flagellum), the maxillary palp (mp), and the proboscis (prob). (B) A region of the flagellum identifying trichoid, basiconic, clavate and coeloconic sensilla, as described by Lewis (1971) and Tangtrakulwanich *et al.* (2011). (C) ScalOrco labelling of nerve cell bodies and dendrites within specific sensilla. Different sensillar types were labelled by anti-Orco, namely basiconic (bas), clavate (cl) and trichoid (tr) sensilla. Scale bar represents 20 µm.

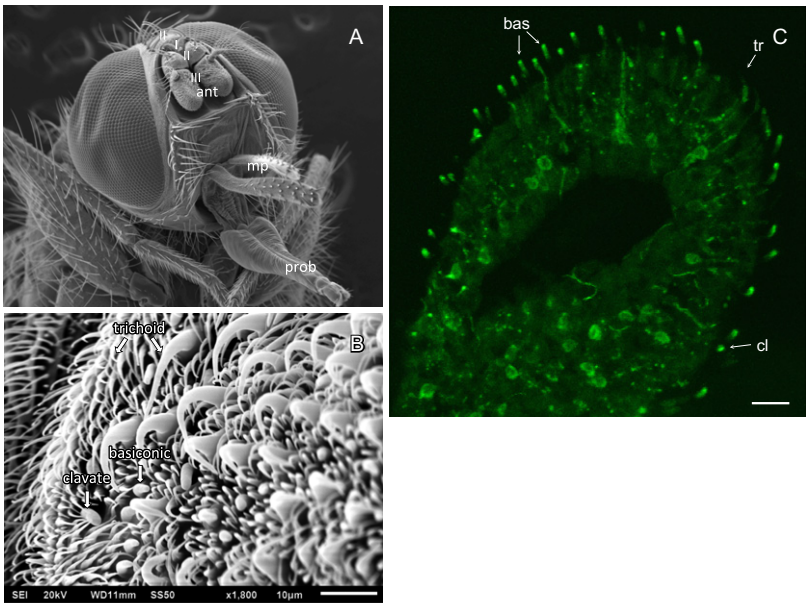

Figure 3. Localization of HirrOrco in *Haematobia irritans* male antenna. Green fluorescence represents anti-Orco detected using an Alexa Fluor 488-conjugated secondary antibody. (A) Scanning electron microscopy of a horn fly head depicting the three antennal (ant) segments (I: scape, II: pedicel, III: flagellum), the maxillary palp (mp), and the proboscis (prob). (B) A region of the flagellum identifying trichoid, basiconic, and clavate sensilla, as described by White & Bay (1980). (C) HirrOrco labelling of nerve cell bodies and dendrites within specific antennal sensilla. Different sensillar types were labelled by anti-Orco, namely basiconic (bas), clavate (cl) and trichoid (tr) sensilla. Scale bar represents 20 µm.

body along the dendritic extension within a specific sensillum, but it was apparent that basiconic, clavate and long trichoid type hairs (sensilla) were labelled in stable fly preparations (Figs 2B, 2C; J.J. Zhu, pers. comm.). Scanning electron micrography observations by both Lewis (1971) and Tangtrakulwanich *et al*. (2011) confirm pores on the surface of basiconic and clavate antennal sensilla, supporting the hypothesis that they have a role in olfaction; however, the authors differed in their assessment of trichoid sensilla, with Tangtrakulwanich *et al*. (2011) assigning them a mechano-receptor function and Lewis (1971) an olfactory function. Anti-Orco labelling of stable fly trichoid sensilla observed in the present study supports an olfactory role, but single-sensillum recordings are desirable to clarify their classification. In horn flies (Fig. 3C), antibody labelling was observed in multiporous thick-walled, thin-walled Ia/Ib, and clavate sensilla, as described by White & Bay (1980). Based on their properties, i.e. length, cuticle thickness, base diameter, these sensilla correspond to what is described in the stable fly as long trichoid, basiconic and clavate sensilla, respectively (Fig. 3B). Insect trichoid sensilla with an olfactory function have been described for mosquitos (Ghaninia *et al*., 2007; Hill *et al*., 2009), *D. melanogaster* (Clyne *et al*., 1997), and the migratory locust (*Locusta migratoria* L.; Cui *et al*., 2011), supporting the results observed. Specificity of antibody labelling was assessed by incubating antennal cryosections with pre-immune serum or with polyclonal antibodies in the presence of the peptide used to generate the anti-serum. In both cases, no distinct cell or sensillum labelling was observed, although there did appear to be some general background (data not shown). Sections were also incubated with the fluorophore-conjugated secondary antibody in the absence of anti-serum; no labelling or background was observed in these sections.

Temporal and spatial expression pattern of Scal\Orco and Hirr\Orco

Using non-quantitative reverse transcriptase-PCR (RT-PCR; Fig. 4), a robust *Scal\Orco* amplification product was detected in pooled antennae/maxillary palps from female and male adults. The transcript was also detected relatively less abundantly in proboscides, thoraces, abdomens and legs of both adult sexes. *Scal\Orco* was detected in most of the immature stages, i.e. 22–24 h embryos, third instar larvae, early (pre-tanning) puparia, and late pharate adults, but no transcript was detected in newly laid (1–3 h) embryos. Conversely, *Hirr\Orco* was expressed in all immature stages evaluated, displaying a relative increase in abundance over time, as evidenced by increasing intensity of the product through the pupal stages (Fig. 4). *Hirr\Orco* was detected most robustly in adult antennae of both sexes and less so in proboscides and maxillary palps. Since horn fly maxillary palps are large compared with those of the stable fly (Fig. 2), they were not pooled with antennae for expression analysis. *Hirr\Orco* expression was relatively faint in abdomens and legs and apparently absent from the thorax of both sexes.

In *Drosophila*, *Orco* is detected in larvae but not in early pupae, and this is coincident with a transition from the larval sensory system to the adult sensory system (Larsson *et al*., 2004; Stocker, 2008). In contrast, there was no apparent decrease in biting fly *Orco* expression during the early (pre-tanning) pupal stages, but a relative decrease in expression for both transcripts was observed

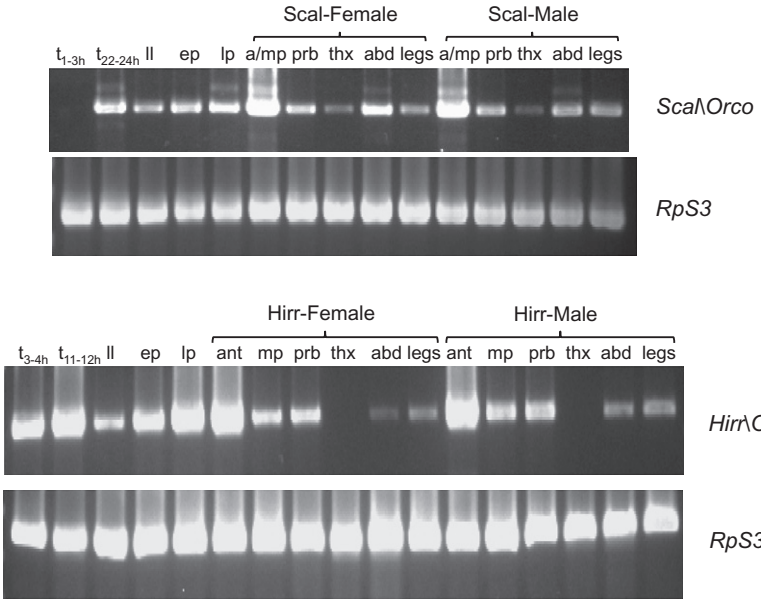

Figure 4. Temporal and spatial expression patterns of *Scal\Orco* (A) and *Hirr\Orco* (B). Temporal expression was evaluated using template synthesized separately from newly laid embryos (t_{1-3h} or t_{3-4h}), pre-hatching embryos (t_{22-24h} or t_{11-12h}), third instar larvae (II), pre-tanning/'early' puparia (ep) and late pharate adults (lp; puparia 2 days prior to adult eclosion). Spatial expression was analysed from female or male pooled antennae-maxillary palp (a-mp; stable fly) or antennae (ant) and maxillary palps (mp) isolated separately (horn fly), proboscis (prb), thorax (thx), abdomen (abd), and legs. Expression of biting fly ribosomal protein S3 (*RpS3*) was used as a positive control for presence of template.

in third instar larvae with subsequent elevated expression during the early and late pupal stages. Detection in the majority of immature stages evaluated suggests the importance of *Orco* throughout biting fly development.

The *Orco* expression pattern observed in biting fly adults supports the hypothesis that it has a role in olfaction, especially given its detection in antennae and maxillary palps, which are classic insect olfactory organs. Apparently equivalent *Orco* expression in female and male antennae was not unexpected since the different types of antennal sensilla are equally represented between the sexes of the stable fly and the horn fly (Lewis, 1971; White & Bay, 1980; Tangtrakulwanich *et al*., 2011). Contact chemoreceptive hairs (taste hairs) have been described on the labellum and tarsi of stable flies (Adams *et al*., 1965; Adams & Forgash, 1966; van der Wolk *et al*., 1984), which indicates that these structures have a gustatory function; however, detection of *Scal\Orco* and *Hirr\Orco* in proboscides and legs suggests either that these structures also have a role in olfaction or that Orco may have an additional, non-olfactory role. *Orco* detection in legs has been reported in several mosquito species (Melo *et al*., 2004; Pitts *et al*., 2004; Xia & Zwiebel, 2006), female fig wasps (Lu *et al*., 2009), the blowfly (*Lucilia sericata*; Wang *et al*., 2012) and *L. migratoria*; Yang *et al*., 2012), yet its expression is absent in legs from the oriental fruit fly (Zheng *et al*., 2012) and several lepidopterans (Malpel *et al*., 2008; Wu *et al*., 2012), further indicating that Orco may have a broader chemosensory role, as suggested by Vosshall *et al*. (2000) and Krieger *et al*. (2003). While *Orco* expression in the proboscides of several mosquitos was attributed to the presence of labial olfactory sensilla (Melo *et al*., 2004; Pitts *et al*., 2004; Kwon *et al*., 2006; Xia & Zwiebel, 2006), comparable sensilla were not observed in head cryosections containing the proboscis of stable flies or horn flies. This does not rule out their existence, however, and additional studies using an electrolabellogram may shed some light on the results obtained in the present study. Detection of *Scal\Orco* in stable fly male and female thoraces was unexpected, especially since *Orco* is not typically detected in this tissue (Pitts *et al*., 2004; Wang *et al*., 2012; Wu *et al*., 2012; Zheng *et al*., 2012) and appears to be absent in thoraces of male and female horn flies. To evaluate whether Orco protein could be detected in these tissues, the insoluble protein fraction was isolated from the heads and thoraces of adult stable flies and horn flies for use in immunoblotting with the anti-Orco antibody. Interestingly, protein was detected in the heads and thoraces of both fly species (Fig. S1) supporting ScalOrco and HirrOrco expression in the thorax and suggesting that the inability to detect *Hirr\Orco* by RT-PCR may be a result of assay sensitivity. Expression of Orco in stable fly thoraces may be an adaptive advantage that enables them to parasitize a broader host range or use more varied ovipositional substrates, similar to that which has been proposed for fig wasps (Lu *et al*., 2009).

Detection of *Hirr\Orco* in the female abdomen corresponds with previous descriptions by Bay *et al*. (1996) of porous sensory pegs located on the anal leaflets at the terminus of the female horn fly ovipositor (Fig. 5B,C); the pores indicate the sensilla have an olfactory function. The anal leaflets also contain numerous, long sensilla that are believed to be mechano-receptors (Bay *et al*., 1996). Scanning electron micrography analysis of the female stable fly ovipositor revealed similar long, grooved sensilla on each anal leaflet that may have a mechanosensory

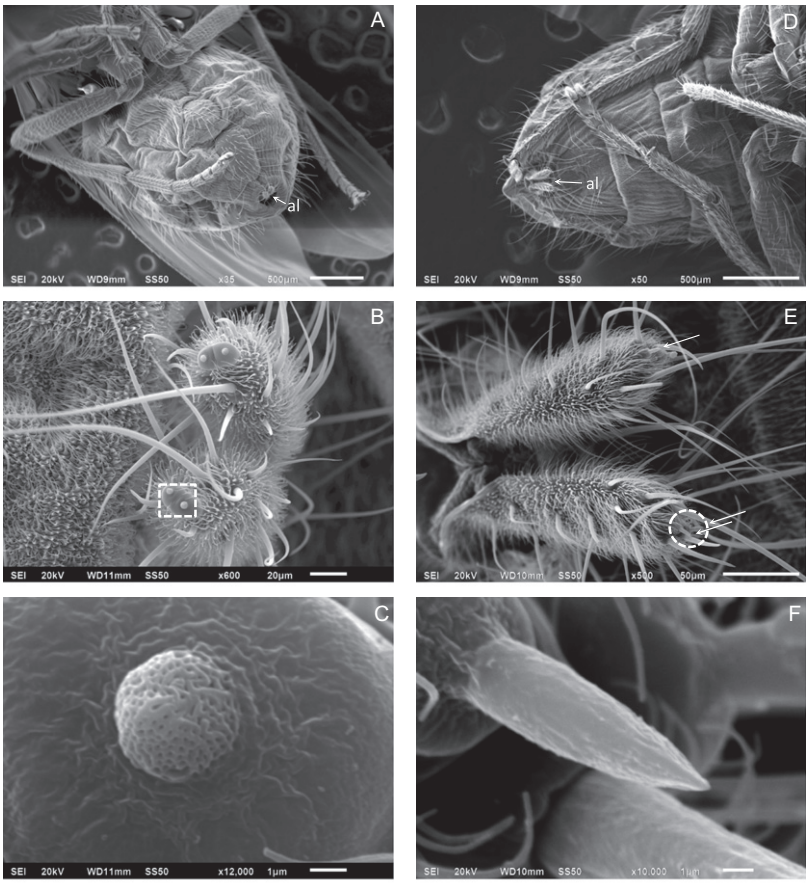

Figure 5. Scanning electron micrographs of the horn fly and stable fly ovipositor. (A) Ventral view of an adult female horn fly with extended terminus showing the paired anal leaflets (al). (B) Horn fly anal leaflets showing the paired multiparous sensory pegs. (C) Higher magnification of the sensory peg [region denoted by a square in (B)]. (D) Ventral view of an adult female stable fly with extended terminus showing the paired anal leaflets (al). (E) Stable fly anal leaflets with pointed sensilla identified by arrows (see text). (F) Higher magnification of the pointed sensillum [region denoted by a circle in (E)].

role; these sensilla surround a distinct, short (~8.8 μm) pointed sensillum situated on an elevated base (Fig. 5E,F) that is located at a position corresponding to the described horn fly sensory pegs. *Scal\Orco* expression was detected by RT-PCR in dissected ovipositors as well as abdomens with the ovipositors removed (Fig. S2); this suggested that the pointed sensillum may have a chemosensory role but also indicated the possibility of other *Scal\Orco*-expressing chemosensory sensilla on the abdomen. The presence of abdominal olfactory sensilla was recently described for the midge, *Culicoides imicola* (Sollai *et al*., 2010), but has not been investigated for biting flies. Several calliphorid fly species also have similar sensory pegs on their anal leaflets (Wallis, 1962; Rice, 1976; Chaiwong *et al*., 2008), emphasizing the importance of olfactory cues to oviposition/ovipositional site selection. Both the stable fly and the horn fly rely on a microbe-rich environment for successful development of the immature stages (Lysyk *et al*., 1999; Romero *et al*., 2006; Talley *et al*., 2009) and may choose a site for oviposition based on the volatiles emitted by the various bacterial species occupying a potential substrate; presence of olfactory sensilla on the abdomen and external genitalia would serve to enhance detection of these olfactory cues.

Collectively, the sequence conservation of ScalOrco and HirrOrco with published insect Orcos, the predicted size and presence of TM domains, and the expression and labelling of ScalOrco and HirrOrco in tissues known to have a role in chemosensation supports the classification of these biting fly sequences as members of the *Orco* gene family (Vosshall & Hansson, 2011). These findings enable future *in vitro* functional characterization of conventional biting fly ORs, providing a means to screen volatile attractant/repellent compounds that may form the basis of supplemental pest control alternatives (Justice *et al*., 2003; van der Goes van Naters & Carlson, 2006).

Experimental procedures

Insect rearing and tissue collection

Stable fly and horn fly specimens used in all experiments were obtained from colonized strains maintained by the Knipling-Bushland US Livestock Insects Research Laboratory (Kerrville, TX, USA) at 27 °C, 60% relative humidity, and a photoperiod of 12 h light:12 h dark. Staged embryos were collected representing newly laid and pre-hatching timepoints (1–3 h and 22–24 h for the stable fly; 3–4 h and 11–12 h for the horn fly). Third instar larvae were collected from both species, as well as newly pupariated (pre-tanning) and late pharate adults (puparia 2 days before adult

emergence). All collected immature stages were frozen on dry ice prior to storage at –80 °C. Antennae, maxillary palps, proboscides, thoraces, abdomens and legs of adult females and males (0–2 days, fed) were dissected from specimens frozen on dry ice, and the tissues were stored separately in RNA*later*®-ICE (Life Technologies, Grand Island, NY, USA) at –20 °C prior to further manipulation.

RNA isolation and cDNA synthesis

Frozen samples of the various immature stages were macerated in TRI Reagent® (Sigma, St. Louis, MO, USA) using a liquid nitrogen-cooled Kontes PELLET PESTLE® (Kimble Chase, Vineland, NJ, USA). Adult samples in RNA*later*®-ICE were centrifuged to collect the tissue, the storage buffer discarded, and the tissues macerated in TRI Reagent® using a liquid nitrogen-cooled Kontes PELLET PESTLE® (Kimble Chase). Subsequent to homogenization, BCP (3-bromopropylchloride; Molecular Research Center, Cincinnati, OH, USA) was used to aid in phase separation and the RNAs were purified on silica matrix spin columns (RNeasy® Mini Kit; Qiagen, Valencia, CA, USA). All total RNAs were DNase-treated using the TURBO DNA-*free*™ system (Life Technologies) following the manufacturer's protocol. First-strand cDNAs were synthesized using equal amounts of total RNA from each source (1 μg), 2.5 μM anchored oligo dT primer (5′-$T_{(20)}$VN -3′), and 0.5 mM dNTP mix denatured at 65 °C for 5 min and then combined with 1 x First Strand Buffer (Life Technologies), 5 mM DTT, 40 U RNaseOUT™ (Life Technologies), and 200 U SuperScript™ III Reverse Transcriptase (Life Technologies) in a total volume of 20 μl. The cDNAs were synthesized at 50 °C for 1.5 h.

Degenerate primed PCR

Degenerate primers were designed based on the amino acid sequences LIFACE (5′- GYTIATHTTYGCITGYGARC-3′) and CQQCQK (5′-GCYTTYTGRCAYTGYTGRCA-3′), which are highly conserved among characterized insect Orco sequences. Oligo-d $T_{(20)}$VN -primed first strand cDNAs synthesized from unfed adult female stable fly and horn fly were used as template for amplification with the following cycling conditions: 94 °C, 2 min; 19 cycles of: 94 °C, 30 s; 55 °C, 40 s; 68 °C, 1 min with a decrease in annealing temperature of 0.5 °C per cycle; and 19 cycles of: 94 °C, 30 s; 45 °C, 40 s; 68 °C, 1 min with a final extension at 68 °C for 3 min. Reactions (50 μl) consisted of 40 mM Tricine-KOH, 15 mM potassium acetate, 3.5 mM magnesium acetate, 3.75 μg/ml bovine serum albumin (BSA), 0.005% Tween 20, 0.005% Nonidet-P40, 0.2 mM dNTPs (Life Technologies), 100 pmol each primer, and 1x Advantage® 2 Polymerase Mix (BD Clontech, Mountain View, CA, USA). Amplified products of the expected size were cloned using the pCR®4-TOPO® TA system (Life Technologies) in TOP10 *Escherichia coli* cells. Transformed cells were plated on Lysogeny broth agar supplemented with kanamycin (50 mg/ml), and plasmid DNA was isolated from 50 isolates using the QIAprep® Spin Miniprep Kit (Qiagen). Plasmid template was used in cycle sequencing with BigDye™ version 3.1 chemistry (Life Technologies), and reactions were analysed on an ABI3130*xl* Genetic Analyzer (Life Technologies).

Isolation of full-length Orco transcripts from ScaI and Hirr (rapid amplification of cDNA ends)

Rapid amplification of cDNA ends (RACE)-ready cDNAs were synthesized separately using stable fly and horn fly unfed adult female total RNA as template in the SMART™ RACE cDNA Amplification Kit (BD Clontech) following the manufacturer's protocol. The 5′ and 3′ RACE for each template was conducted using an ScaI or Hirr *Orco*-specific primer in a primary round of cycling followed by a nested reaction using a transcript-specific nested primer, both in combination with relevant commercial adapter primers UPM (primary) or NUPM (nested). Primers used in 5′ and 3′ stable fly RACE reactions were ScalOr83b-Rev4 (primary 5′; 5′- GCCCAAGTAGCCGATCAC-3′), ScalOr83b-Rev5 (nested 5′; 5′- TTGGTTGCCTGGTACGC-3′), ScalOr83b-Fwd1 (primary 3′; 5′-CAGTTGCAGCATTTGAAGGG-3′), and ScalOr83b-Fwd2 (nested 3′; 5′- CATAATGAAACCTCTGATGGAGCTG-3′). Primers used in 5′ and 3′ horn fly RACE reactions were HiOr83b-Rev1 (primary 5′; 5′-GCCCAAGTAGCCGATCAC-3′), HiOr83b-Rev2 (nested 5′; 5′- AGTAAACGCATAGACATCCACACC-3′), HiOr83b-Fwd1 (primary 3′; 5′- AGCATCACTGGACACCTACCG -3′) and HiOr83b-Fwd2 (nested 3′; 5′- ACCTAATTCGGCTGCCTT -3′). Fragments of interest were cloned and sequenced as described above. A 5.8 kb stable fly genomic sequence comprising exons that are spliced to encode amino acids 1–342 of ScalOrco was obtained by primer walking using the Genome Walker system (Clontech). The sequence comprises four exons and four introns, the last intron of which was >4 kb in length. The genomic sequence downstream of this region was not obtained. Further, primer pair HirrOr83b F5/R4 was used to amplify horn fly genomic DNA revealing the presence of a 208-bp intron, the location of which is shared with intron 1 of the *ScaI**Orco* partial genomic sequence. These data supported the use of selected primers for gene expression studies that could assist in distinguishing the presence of genomic DNA contamination, if present.

Semi-quantitative evaluation of ScalOrco and HirrOrco expression

First-strand cDNAs (equivalent to the amount synthesized from 20 ng total RNA) were used as template in 20 μl RT-PCR reactions consisting of 40 mM Tricine-KOH, 15 mM potassium acetate, 3.5 mM magnesium acetate, 3.75 μg/ml BSA, 0.005% Tween 20, 0.005% Nonidet-P40, 0.2 mM dNTPs (Life Technologies), 5 pmol each primer, and 1x Advantage® 2 Polymerase Mix (BD Clontech). Cycling conditions consisted of: 95 °C, 2 min; 35 cycles of 95 °C, 30 s; 65 °C, 30 s; 68 °C, 30 s with final extension at 68 °C, 2 min. Primer combinations used were ScOr83b Fwd4/ScOr83b R7 (expected size: 1090 bp) and HiOr83b-ATG/HiOr83b-Rev2 (expected size: 1173 bp); these were designed to span introns, providing a means to detect genomic DNA contamination if present. The RpS3 gene was amplified as an internal control for the presence of template using primer pair RpS3 Fwd/Rev ($T_{annealing}$: 63 °C) for both the stable fly and horn fly samples. Primer sequences used were as follows: ScalOr83b-Fwd4: 5′-GGTGCAGTTCGCCTTGA -3′; ScalOr83b-R7: 5′-CAGCTTCCATGACCGATG-3′; HiOr83b-ATG: 5′ -ATGACGAGTATGCAACCCACC-3′; HiOr83b-Rev2: 5′-AGT AAACGCATAGACATCCACACC-3′; RpS3-Fwd: 5′ -CGTTCTCT GCGAGTCGTATG-3′; RpS3-Rev: 5′-TTAATGAGCAGCAGC TTCATCA-3′. A 'no template' control (water only) was included in all RT-PCR amplification experiments.

Antibody production and immunohistochemistry

A 15 amino acid peptide (KSELINNEEKEPVN), located within the predicted intracellular loop connecting TM4 and TM5, was

synthesized, conjugated to keyhole limpet hemocyanin, and used to generate sequence-specific, peptide-affinity purified polyclonal antibodies in rabbits (Genscript, Piscataway NJ, USA).

Adult stable flies and horn flies (0–2 days old, fed) were fixed for 30 min in 4% paraformaldehyde in phosphate-buffered saline (PBS) with 0.1% Triton X (PBSTx), washed with PBS for a total of 30 min (10 min/wash), and transferred to 25% sucrose overnight. Flies that sank to the bottom of the container were loaded onto a Fly Collar (Genessee Scientific, San Diego, CA, USA) to properly orient the heads. The heads were embedded in Tissue-Tek O.C.T. embedding medium (Sakura Finetek, Torrance, CA, USA), and blocks were wrapped in aluminum foil and stored at −20 °C prior to sectioning. Head cryosections (10–14 μm) obtained using a Leica CM1800 cryostat (Leica Microsystems, Buffalo Grove, IL, USA) were thaw-mounted on Polysine® microscope slides (Erie Scientific, Portsmouth, NH, USA) and air-dried at room temperature for 30 min before either using immediately or storing at −20 °C. Slides were incubated in fixative (4% paraformaldehyde in PBSTx) for 30 min, rinsed with PBSTx for a total of 30 min (10 min/wash), and blocked for 1 h at room temperature in blocking solution (PBSTx with 5% normal donkey serum). Primary anti-sera was diluted 1:2500 in blocking solution, dispensed onto sections and incubated at 4 °C overnight. Slides were washed in PBSTx for a total of 30 min (10 min/wash) and incubated at room temperature for 2 h in donkey anti-rabbit Alexa 488 antibody (Life Technologies) diluted 1:1000 in blocking solution. Slides were washed for a total of 30 min in PBSTx (10 min/wash) and coverslips were mounted in 80% glycerol. Sections were visualized on an LSM 510 Meta confocal microscope (Zeiss, Thornwood, NY, USA), and Adobe Photoshop Elements version 10 was used to adjust image brightness/contrast.

Sequence analyses and sequence accession numbers

Insect Orco amino acid sequences were aligned using Clustal W (Larkin *et al*., 2007), and a neighbour-joining tree (unrooted) was calculated with 1000 bootstrap replicates using the MEGA-5 program (Tamura *et al*., 2007). The publicly available TopPred (Claros and von Heijne, 1994; http://www.mobyle.pasteur.fr), TMHMM server version 2.0 (Krogh *et al*., 2001; http://www.cbs.dtu.dk/services/TMHMM/), and TMpred (Hofmann & Stoffel, 1993; http://ch.embnet.org/software/TMPRED_form.html) programs were used to predict locations of TM domains within Scal and HirrOrco. The GenBank accession numbers for the genes described in this study are: *Scal\Orco* transcript: EU622914, *Scal\Orco* partial genomic sequence: JX996042 *Hirr\Orco* transcript: EU622915, *Hirr\Orco* partial genomic sequence: JX996043.

Scanning electron microscopy

Whole adult stable fly and horn fly specimens (female and male, fed) were incubated in fixative (4% formaldehydye, 1% gluteraldehyde) for at least 24 h, after which they were rinsed in 0.1 M phosphate buffer and incubated in 1% Zetterquist's Osmium for 30 min. The fly specimens were rinsed in Zetterquist's buffer for 2 min and dehydrated in a series of ethanol washes: 70% for 45 min, 95% for 45 min, and 100% for 40 min. The specimens were then immersed in hexamethyldisilizane for 5 min, air dried in a dessicator, mounted on aluminum stubs and sputter coated with gold palladium. Specimens were observed using a JSM-6610LV scanning electron microscope (JEOL Ltd, Tokyo, Japan) at the Pathology Electron Microscopy Facility, University of Texas Health Science Center (San Antonio, TX, USA).

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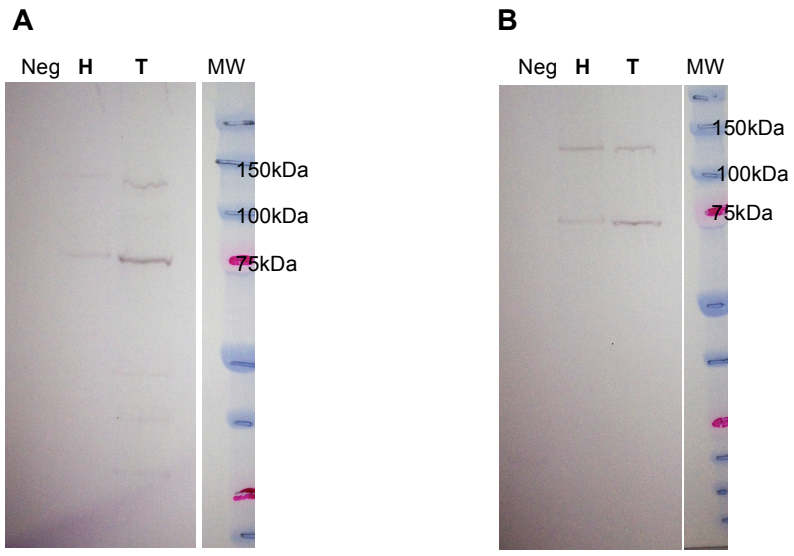
Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/imb.12009

Figure S1. Detection of ScalOrco (A) and HirrOrco (A) by immunoblotting. Insoluble protein fractions were isolated separately from stable fly and horn fly heads (H) and thoraces (T). The anti-Orco antibody labelled two distinct fragments in each lane that were 75 kDa and ~130 kDa in size, possibly representing Orco protein monomer and OR-Orco dimers, respectively. The estimated molecular mass of ScOrco and Hirr Orco is ~54 kDa, suggesting a gel migration shift that is typical of membrane protein fractions (Rath *et al*., 2009). Protein equivalent to a single head or thorax was resolved by polyacrylamide gel electrophoresis on a NuPAGE® 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) in MOPS/SDS running buffer (Invitrogen) under reducing conditions. The Precision Plus Protein™ Standard (MW; Bio-Rad Laboratories, Hercules, CA, USA) was included, and a recombinant tick protein was resolved alongside the fractions as an irrelevant negative control (Neg). Proteins were transferred to a polyvinylidene difluoride membrane using a TransBlot™ SD Semi-Dry Transfer Cell (Bio-Rad Laboratories), washed in phosphate-buffered saline/0.3% Tween 20 (PBSTw20), blocked in 10% goat milk, and incubated in biting fly anti-Orco (1:500) at 4 °C overnight. Blots were rinsed in PBS-Tw20, incubated in horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:20,000; Bio-Rad Laboratories), and developed for 30 min using an amplified Opti-4CN™ substrate colorimetric detection assay (Bio-Rad Laboratories). Three faint, lower molecular mass bands developed in the stable fly thorax lane after 20 min of incubation in developer.

Figure S2. Expression of *ScalOrco* in female abdomen and ovipositor. Presence of the transcript was evaluated using primer pair ScOr83b-Fwd4/R7 and template synthesized separately from abdomens (Abd), dissected ovipositors (Ovip) and abdomens with the ovipositors removed (Abd no Ovip). The transcript was detected in all templates evaluated. Expression of biting fly ribosomal protein S3 (*RpS3*) was used as a positive control for the presence of template.



Supplemental Figure 1. Detection of ScalOrco (A) and HirrOrco (A) by immunoblotting. Insoluble protein fractions were isolated separately from stable fly and horn fly heads (H) and thoraces (T). The anti-Orco antibody labeled two distinct fragments in each lane that were 75 kDa and ~130kDa in size possibly representing Orco protein monomer and Or-Orco dimers, respectively. The estimated molecular mass of ScOrco and Hirr Orco is ~54kDa, suggesting a gel migration shift that is typical of membrane protein fractions (Rath et al., 2009). Protein equivalent to a single head or thorax was resolved by PAGE on a NuPAGE® 4-12% Bis-Tris gel (Invitrogen) in MOPS/SDS running buffer (Invitrogen) under reducing conditions. The Precision Plus Protein™ Standard (MW; Bio-Rad Laboratories, Hercules, CA) was included, and a recombinant tick protein was resolved alongside the fractions as an irrelevant negative control (Neg). Proteins were transferred to a PVDF membrane using a TransBlot™ SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA), washed in PBS/0.3% Tween 20 (PBS-Tw20), blocked in 10% goat milk, and incubated in biting fly anti-Orco (1:500) at 4 °C overnight. Blots were rinsed in PBS-Tw20, incubated in HRP-conjugated goat anti-rabbit secondary antibody (1:20,000; Bio-Rad Laboratories), and developed for 30 mins using an amplified Opti-4CN™ substrate colorimetric detection assay (Bio-Rad Laboratories). Three faint, lower molecular mass bands developed in the stable fly thorax lane after 20 mins of incubation in developer.

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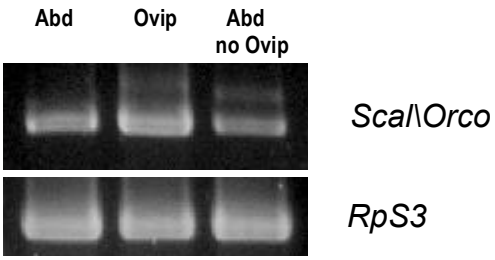

Supplemental Figure 2. Expression of *Scal\Orco* in female abdomen and ovipositor. Presence of the transcript was evaluated using primer pair ScOr83b-Fwd4/R7 and template synthesized separately from abdomens (Abd), dissected ovipositors (Ovip) and abdomens with the ovipositors removed (Abd no Ovip). The transcript was detected in all templates evaluated. Expression of biting fly ribosomal protein S3 (*RpS3*) was used as a positive control for the presence of template.