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The maxillary palp of *Aedes aegypti*, a model of multisensory integration

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ABSTRACT

Female yellow-fever mosquitoes, *Aedes aegypti,* are obligate blood-feeders and vectors of the pathogens that cause dengue fever, yellow fever and Chikungunya. This feeding behavior concludes a series of multisensory events guiding the mosquito to its host from a distance. The antennae and maxillary palps play a major role in host detection and other sensory-mediated behaviors. Compared to the antennae, the maxillary palps are a relatively simple organ and thus an attractive model for exploration of the neuromolecular networks underlying chemo- and mechanosensation. In this study, we surveyed the expressed genetic components and examined their potential involvement with these sensory modalities. Using Illumina sequencing, we identified the transcriptome of the maxillary palps of physiologically mature female *Ae. aegypti*. Genes expressed in the maxillary palps included those involved in sensory reception, signal transduction and neuromodulation. In addition to previously reported chemosensory genes, we identified candidate transcripts potentially involved in mechanosensation and thermosensation. This survey lays the groundwork to explore sensory networks in an insect appendage. The identification of genes involved in thermosensation provides prospective molecular targets for the development of chemicals aimed at disrupting the behavior of this medically important insect.

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1. Introduction

The yellow-fever mosquito, *Aedes aegypti* (Diptera: Culicidae), is both a nuisance and a threat to public health due to its notorious ability to bite humans and spread a variety of diseases such as Chikungunya, yellow fever and dengue fever (Bhatt et al., 2013). This feeding behavior results from a unique combination of morphological, physiological and genetic characteristics of the mosquito sensory system including vision, chemosensation, thermosensation, mechanosensation and hygrosensation. These sensory functions are carried out by a complex peripheral sensory system mainly deployed on the head of the mosquito and consisting of the compound eyes, antennae, mouthparts and maxillary palps (Fig. 1A).

In comparison to the antennae and mouthparts, the maxillary palps of female *Ae. aegypti* are simple in their structure and function. The maxillary palps are composed of five segments (Fig. 1A) with five surface cuticular structures, namely microtrichia, scales, sensilla chaetica, capitate sensilla basiconica (McIver, 1982) and a possible sensillum campaniformia (Fig. 1B and C). With the exception of scales, this organization is similar to the maxillary palps of *Drosophila melanogaster* (de Bruyne et al., 1999; Stocker, 1994). The microtrichia are distributed throughout the surface of the maxillary palps, while scales cover most of its surface except for the medial and ventral areas where the paired maxillary palps meet. The maxillary palps are mobile appendages that vibrate during probing and initial phase of blood-feeding (McIver and Charlton, 1970). Both the scales and microtrichia are non-innervated and thus have no sensory function. Most of the sensilla chaetica (McIver, 1982) and all the capitate sensilla basiconica (McIver, 1972) are located on the dorso-lateral side of the fourth segment and are innervated by approximately 100 sensory neurons (McIver, 1982). The sensilla chaetica are non-porous mechanosensory structures of various lengths innervated by one sensory neuron (Fig. 1C). Since they do not touch the skin surface during blood-feeding, these sensilla likely provide spatial, airflow or movement information. The capitate sensilla basiconica are porous and house 3 chemosensory neurons named “A”, “B” and “C”

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14. ABSTRACT

Female yellow-fever mosquitoes, Aedes aegypti, are obligate blood-feeders and vectors of the pathogens that cause dengue fever, yellow fever and Chikungunya. This feeding behavior concludes a series of multisensory events guiding the mosquito to its host from a distance. The antennae and maxillary palps play a major role in host detection and other sensory-mediated behaviors. Compared to the antennae, the maxillary palps are a relatively simple organ and thus an attractive model for exploration of the neuromolecular networks underlying chemo- and mechanosensation. In this study, we surveyed the expressed genetic components and examined their potential involvement with these sensory modalities. Using Illumina sequencing, we identified the transcriptome of the maxillary palps of physiologically mature female Ae. aegypti. Genes expressed in the maxillary palps included those involved in sensory reception, signal transduction and neuromodulation. In addition to previously reported chemosensory genes, we identified candidate transcripts potentially involved in mechanosensation and thermosensation. This survey lays the groundwork to explore sensory networks in an insect appendage. The identification of genes involved in thermosensation provides prospective molecular targets for the development of chemicals aimed at disrupting the behavior of this medically important insect.

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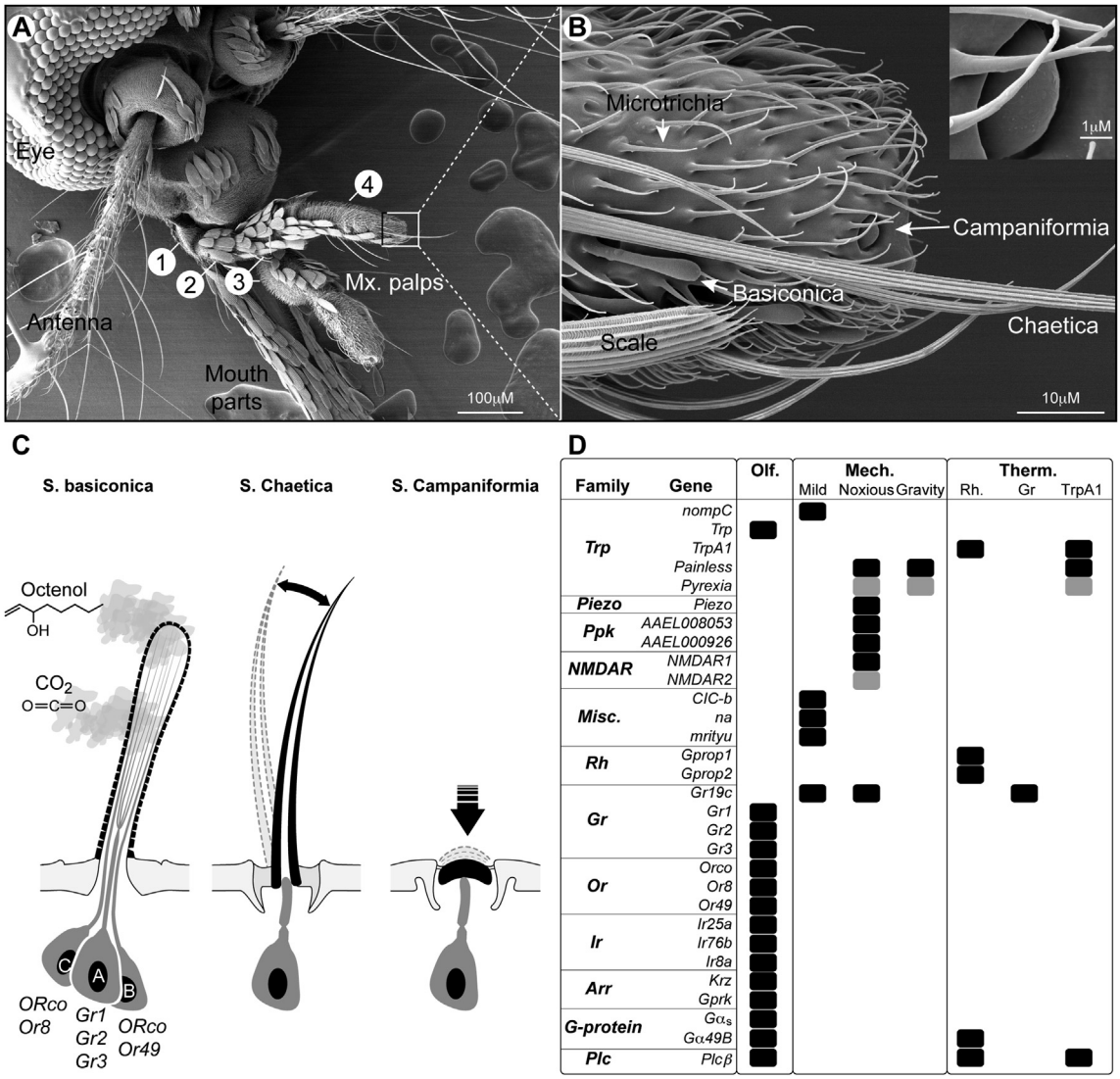


Fig. 1. Chemosensory structures and genes of the maxillary palp. (A) The maxillary palps are composed of five segments of which only 4 are shown. The small 5th segment is fragile and generally lost during specimen preparation for microscopy. Segment 4 houses most of the sensory structures found on the palp. The box indicates the location of the sensillum campaniformia. (B) The distal end of the fourth segment exhibits all the morphological structures present on the palp including the scales, microtrichia, capitate sensilla basiconica and the sensillum campaniformia (see inset). (C) Three innervated sensory organs are found on the palp of *Ae. aegypti*. Capitate sensilla basiconica are porous hairs, which house three chemosensory neurons sensitive to octenol, CO_2 and several human skin odorants. These neurons express odorant (*Ors*) and gustatory receptors (*Grs*). Sensilla chaetica and campaniformia are mechanoreceptors containing one sensory neuron. (D) Gene transcripts expressed in the maxillary palps may contribute to olfaction (Olf.), mechanosensation (Mech.) and thermosensation (Therm.). In flies, thermosensation is dependent on a Rhodopsin (Rh.), a gustatory receptor (Gr) and a transient receptor potential channel (TrpA1) pathway. These transcripts include transient receptor potential channel (*Trp*), *Piezo*, Pickpocket (*Ppk*), N-methyl-D-aspartate receptor (*NmdaR*), Rhodopsin (*Rh*), Gustatory receptor (*Gr*), Odorant receptor (*Or*), Ionotropic receptor (*Ir*), Arrestin (*Arr*), G-protein (*Gp*), Phospholipase C (*Plc*), chloride channel-b (*ClC-b*), narrow abdomen (*na*) and *mrityu*. Black and shaded boxes indicate *D. melanogaster* function. Black boxes also indicate *Ae. aegypti* expression and inferred function.

based on the amplitude of their action potentials (Fig. 1C). Neuron "A" produces the largest amplitude action potential and responds to CO_2 (Kellogg, 1970; Grant et al., 1995) via the activation of at least two gustatory receptors (GR1 & GR3) (Erdelyan et al., 2011) and to human skin odorants possibly also mediated by gustatory receptors (Tauxe et al., 2013). The function of neuron "B", which produces intermediate size action potentials, is unknown as its cognate stimulus remains to be identified. Neuron "C" is activated by the mosquito attractant (*R*)-(−)-octen-3-ol (Grant and Dickens, 2011), a response mediated by the odorant receptor complex OR8-ORco (Bohbot and Dickens, 2009) (Fig. 1C). Consistent with other mosquito species (Seenivasagan et al., 2009; McIver and Siemicki, 1975), we have identified a candidate sensillum campaniformia underneath a layer of scales on the disto-lateral end of segment 4 in *Ae. aegypti* (Fig. 1B). In insects, sensillum campaniformia house sensory neurons that function as proprioceptors responsive to deformation of the cuticle (Keil, 1997).

The chemosensory proteins present in the maxillary palps are encoded by members of large families of receptor and associated genes. Previous studies have surveyed one or a restricted number of sensory gene families in the genome (Hill et al., 2002; Zhou et al., 2008; Kent et al., 2008; Pelletier and Leal, 2011; Croset et al., 2010; Manoharan et al., 2013; Sparks et al., 2013) or in specific appendages (Vosshall et al., 1999; Rutzler et al., 2006; Bohbot et al., 2007; Pitts et al., 2011; Rinker et al., 2013; Shiao et al., 2013) of dipterans but none have carried out comprehensive analyses of

global sensory gene expression in specific appendages. Now, we have sequenced the maxillary palp transcriptome and focused on the expression of a gene set comprised of 503 genes belonging to 18 gene families whose functions are inferred from studies in *D. melanogaster*, including genes involved in sensory reception, sensory signal transduction and neuromodulation. Drawing from knowledge on the genetic networks involved in sensory function in *D. melanogaster* in combination with phylogenetic analyses, we have identified candidate sensory genes, their relationship to each other, and suggested their potential roles in olfaction, mechanosensation and thermosensation. These discoveries raise questions about the sensory functions of the maxillary palp and provide new candidates for future sensory-mediated behavioral disruption.

2. Materials and methods

2.1. Insects and tissue collection

Six day old adult female *Ae. aegypti* mosquitoes (Orlando strain, 1952 Florida) were kept at 27 °C and 70% relative humidity with a 12:12 light–dark cycle. Genders were mixed together and fed on a 10% sucrose solution. Maxillary palps were collected on dry ice until RNA extraction.

2.2. RNA isolation and cDNA synthesis

Maxillary palps were manually disrupted using TRIzol® (Invitrogen Life Technologies, Carlsbad CA, USA) and total RNA was purified according to the manufacturer's guidelines. Five hundred and ten maxillary palps were collected for RNA-sequencing and 158 maxillary palps were used for qRT-PCR measurements. Total RNA concentrations were measured using a Nanodrop 1000 spectrophotometer and sample quality was determined at 260 nm/280 nm. For Next-Generation Sequencing, RNA samples were sent to the Genomic Services Lab at Hudson Alpha Institute for Biotechnology (Huntsville, Alabama). Isolation of Messenger RNA and cDNA synthesis was prepared using the Illumina® TruSeqTM RNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA). The library was sequenced on an Illumina HiSeq2000 to generate 50 bp paired-end reads (Fig. 1). For qPCR, cDNA syntheses were carried out using the Superscript® III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

2.3. Quantitative real-time PCR

Seven chemosensory and one housekeeping genes were selected to validate RNA-seq by RT-qPCR. Primers were designed (Primer-BLAST Primer Designing tool, NCBI) to span exon-intron boundaries whenever possible and to generate amplicon ranging from 125 bp to 185 bp (Table S2). cDNA quantification was carried out on an iCycler iQ™ thermocycler (Bio-Rad, Hercules, CA) using the KiCqStart® SYBR® Green qPCR ReadyMix™ iQ kit (Sigma–Aldrich, St Louis, Mo, USA). Reactions (20 μL) consisted of template cDNA, 1X reaction buffer including SYBR Green dye and Taq DNA polymerase (Sigma, St. Louis, MO, USA) and 0.3 μM of forward and reverse primers in a 20 μL volume reaction. Cycling conditions included an initial 30 s denaturation step at 95 °C followed by 45 cycles at 95 °C for 10 s, 56 °C for 15 s and 68 °C for 30 s. The size and purity of PCR amplicons were determined on a 2% TAE agarose gel. Transcripts within these bands were also validated by direct sequencing (Macrogen, Rockville, MD). Threshold values (C_T) were calculated using the Bio-Rad iQ5 Optical System Software (Bio-Rad, Hercules, CA) based on three biological and three technical replicates generating 9 measurements per biological sample. We used the housekeeping Lysosomal Aspartic Protease gene (*LAsP*) (Dittmer and Raikhel, 1997) as our reference gene for relative expression analysis.

The efficiency of each primer set was calculated from the standard curve slope according to the equation $E = 10^{(-1/\text{slope})}$ (Pfaffl, 2001) (Table S2). The standard curve was generated by RT-qPCR using 10 fold serial dilutions of the cDNA template in order to encompass the sample C_T values. Gene quantification for each target gene was determined using the $E_{\text{target}}^{-(\text{CT[target]}-\text{CT[reference]})}$ formula. Gene quantification methods were compared by fitting the least-squares linear regression of RNA-seq onto RT-qPCR: RNA-seq $= y_0 + a$ (qPCR) where "y_0" (intercept) is −1.34 and "a" (slope) is 1.5 (Supplemental Fig. 1).

2.4. RNA-seq profiling

Illumina read files were mapped onto the *Ae. aegypti* transcriptome (VectorBase version 1.3). Processing and analysis of the Illumina files was carried out as described previously (Bohbot et al., 2013). Transcript expression levels were reported in Reads per Kilo-Base per Million reads mapped (RPKM) (Table S1). Defining expression cutoff in the context of RNA-seq is arbitrary (Wagner et al., 2013). RPKM cutoff values typically range from 1 (Hebenstreit et al., 2011) to 3 (Lee et al., 2011). Our first concern was to avoid type 1 errors or false positives, since very low transcript expression were not our interest. We therefore used a conservative RPKM value of 3 as our criterion to define transcript expression.

2.5. Gene curation

We used previously reported gene annotations to catalogue the *Ae. aegypti Or* (Bohbot et al., 2007), *Gr* (Kent et al., 2008), *Ir* (Croset et al., 2010) and *Obp* (Manoharan et al., 2013) genes. All the other genes reported in this study were identified by BLAST search of the NCBI and VectorBase databases using *D. melanogaster* and *Ae. aegypti* genes as query. We used OrthoDB (http://cegg.unige.ch/orthodb7) to identify additional *Ae. aegypti* genes using *D. melanogaster* and *Ae. aegypti* genes as query. In three instances, we found redundant annotations in VectorBase, which we excluded from phylogenetic analyses. AAEL001268 is a fragment of the larger AAEL009419 transcript and AAEL011034 and AAEL013378, which are located on 2 different supercontigs, encode almost identical TRP channels except for an extra 8 N-terminal amino acids in the latter case. AAEL012760 is a concatenation of two Ppk encoding genes (AAEL000547 and AAEL013573) and was thus also removed from our analysis.

2.6. Protein sequence analyses

Peptide sequence comparisons were generated from multiple sequence alignments using Clustal Omega with default settings (Sievers et al., 2011). Amino-acid sequence identity was determined using compositionally adjusted substitution matrices (Altschul et al., 2005). We used the neighbor-joining statistical method for phylogeny reconstruction (Molecular Evolutionary Genetics Analysis software version 5.1.2) (Tamura et al., 2011). Evolutionary distances were estimated with a pairwise distance method and gaps/missing data were handled with the pairwise deletion method. Node robustness was assessed using 1000 bootstrap replications. Nodes for which bootstrap support were below 50% were collapsed.

3. Results and discussion

3.1. Transcriptome sequencing and read mapping

We previously reported the expression levels of a subset of *Or* and *Gr* genes in the maxillary palps of female *Ae. aegypti* in relation

to their potential role in sensory physiology and behavior and hypothesized that presynaptic factors modulated sensory activity (Bohbot et al., 2013). In order to evaluate this theory, we sequenced the maxillary palp transcriptome of 6-day-old female *Ae. aegypti* mosquitoes and searched for additional putative sensory and neuromodulatory genes (Fig. 2A). Over 90 million 50 base-pair reads were generated, of which 84.7% were successfully mapped to the *Ae. aegypti* gene set in VectorBase (AaegL1.3). 10,796 of the total 18,760 predicted transcripts were expressed well above background (RPKM values equal to or above 3, Fig. 2B) (explained 'Material and Methods'). These levels of coverage and gene count were consistent with a previous study on the maxillary palps of female *Anopheles gambiae* carried out using the same Illumina sequencing technology (Pitts et al., 2011). Our study subsequently focused on 503 genes belonging to 15 gene families involved with chemosensory signaling and neuromodulation (Fig. 2A).

3.2. qRTPCR validation of mRNA sequencing

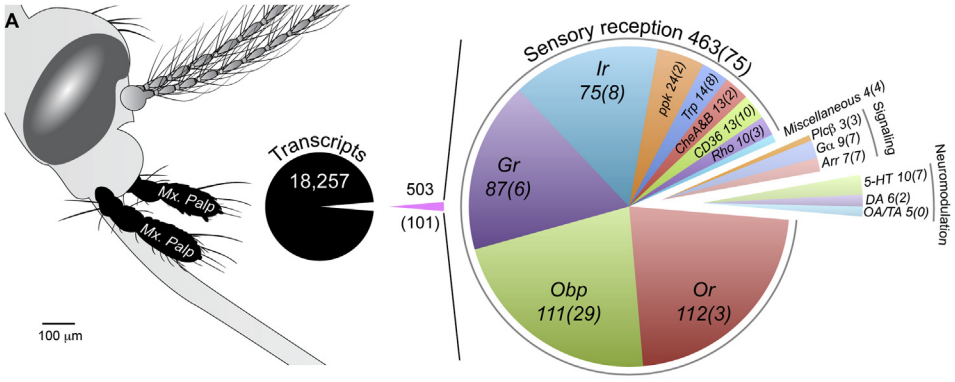
To validate the RNA sequencing analysis, we measured the expression levels of 8 transcripts by quantitative Real-Time PCR (Supplemental Fig. 1). Selected transcripts were pooled from three olfactory receptors gene families (*Ors*, *Grs* and *Irs*) and the housekeeping gene *LAsP* was used to calibrate. Pairwise comparisons of the levels of 8 transcripts, as determined by RNA-seq and validated by quantitative Real-Time PCR, demonstrated similarity (Supplemental Fig. 1A). To further explore this pairwise relationship, we calculated the least square regression line that best fitted the data and observed a positive relationship between the two gene quantification methods (Supplemental Fig. 1B) suggesting that these two techniques are closely related and thus concordant in their estimation of gene expression.

3.3. Chemosensation

Three receptor gene families are known to have a chemosensory role in insects: odorant receptors (*Ors*), gustatory receptors (*Grs*) and ionotropic receptors (*Irs*) (Fig. 1D). ORs function as ligand-gated ion channels that are comprised of a conventional ligand-sensing subunit and an obligatory co-receptor named ORco (Larsson et al., 2004; Vosshall and Hansson, 2011). The operative mechanism of GRs remains elusive, usually requiring co-expression of at least one other GR in gustatory receptor neurons (Dahanukar et al., 2007; Weiss et al., 2011), while in some instances functioning alone (Sato et al., 2011; Miyamoto et al., 2012). GRs have been implicated in the detection of CO_2 (Jones et al., 2007; Kwon et al., 2007), sugars (Dahanukar et al., 2007; Jiao et al., 2008), bitter compounds (Weiss et al., 2011) and contact pheromones (Watanabe et al., 2011). Much like ORs, functional IRs are heteromers comprised of a ligand-binding subunit associated with one or multiple co-receptors (Abuin et al., 2011). Specifically, the expression of the *Ir8a*, *Ir25a* and *Ir76b* co-receptors in a given tissue is indication of IR-mediated detection of acids and amines (Benton et al., 2006).

As reported previously (Bohbot et al., 2007), *Orco*, *Or8* and *Or49* are abundantly expressed in the maxillary palps of *Ae. aegypti* (Fig. 3). *Or6* and *Or99* displayed RPKM values above 2, which may be functionally significant but is below our expression threshold of 3 RPKM (see Materials and Methods section). These genes had not been detected in this appendage using classic PCR amplification (Bohbot et al., 2007). *Or8* and *Orco* encode two proteins both required for octenol sensitivity, specifically the (*R*)-enantiomer of 1-octen-3-ol (Bohbot and Dickens, 2009; Lu et al., 2007). While the presence of *Or49* in the maxillary palps has previously been reported (Bohbot et al., 2007), its ligand sensitivities remain unknown.

Until now, only three *Gr* genes (*Gr1*, *Gr2* and *Gr3*) have been described in the maxillary palp of *Ae. aegypti* (Kent et al., 2008; Erdelyan et al., 2011; Bohbot et al., 2013), two of which (*Gr1* and *Gr3*) are required for normal detection of CO_2. This mirrors CO_2 detection in *D. melanogaster*, as orthologous genes *Gr21a* (Aaeg*Gr1*) and *Gr63a* (Aaeg*Gr3*) are required for CO_2 detection by olfactory neurons in the antennae. In addition to these receptor components, transient receptor potential channel genes (*Trp* and *Trpl*) (Badsha et al., 2012), a phospholipase gene (*Plc21c*) and G-protein subunit alpha 49B (*Gα49B*) (Yao and Carlson, 2010) affect sensitivity to CO_2 in these *D. melanogaster* olfactory neurons. *Trp* channel genes are highly conserved cation channels that are involved in many different sensory contexts in insects (Fowler and Montell, 2013)



B

Tissue	Total reads	Aligned reads (%)	High-quality aligned reads (%)	Transcripts > 3 RPKM (%) (18,760 transcripts total)	Mean RPKM
Female palps	91,110,802	77,178,110 (84.7)	66,528,540 (73.0)	10,796 (57.5)	28.24

Fig. 2. Transcriptome survey of sensory genes in the maxillary palps of female *Ae. aegypti*. (A) 503 genes belonging to over 15 gene families involved in sensory reception (Odorant receptor, *Or*; Odorant-binding protein, *Obp*; Gustatory receptor, *Gr*; Ionotropic receptor, *Ir*; Pickpocket, *ppk*; Transient receptor protein channel, *Trp*; Chemosensory, *CheA* and *CheB*; Cluster of Differentiation 36, *CD36*; Rhodopsin, *Rho* and other 4 miscellaneous genes), signal transduction (Phospholipase C, *Plcβ*; Guanine nucleotide binding protein alpha, G*α* and Arrestin, *Arr*) and neuromodulation (Serotonin, *5-HT*; Dopamine, *DA*, Octopamine, *OA* and Tyramine, *TA*) were surveyed in the maxillary palp (Mx. Palp) of *Ae. aegypti*. Numbers of studied and expressed genes (in parentheses) are indicated beside each gene family. (B) Mapping characteristics and overall expression information of the whole transcriptome library.

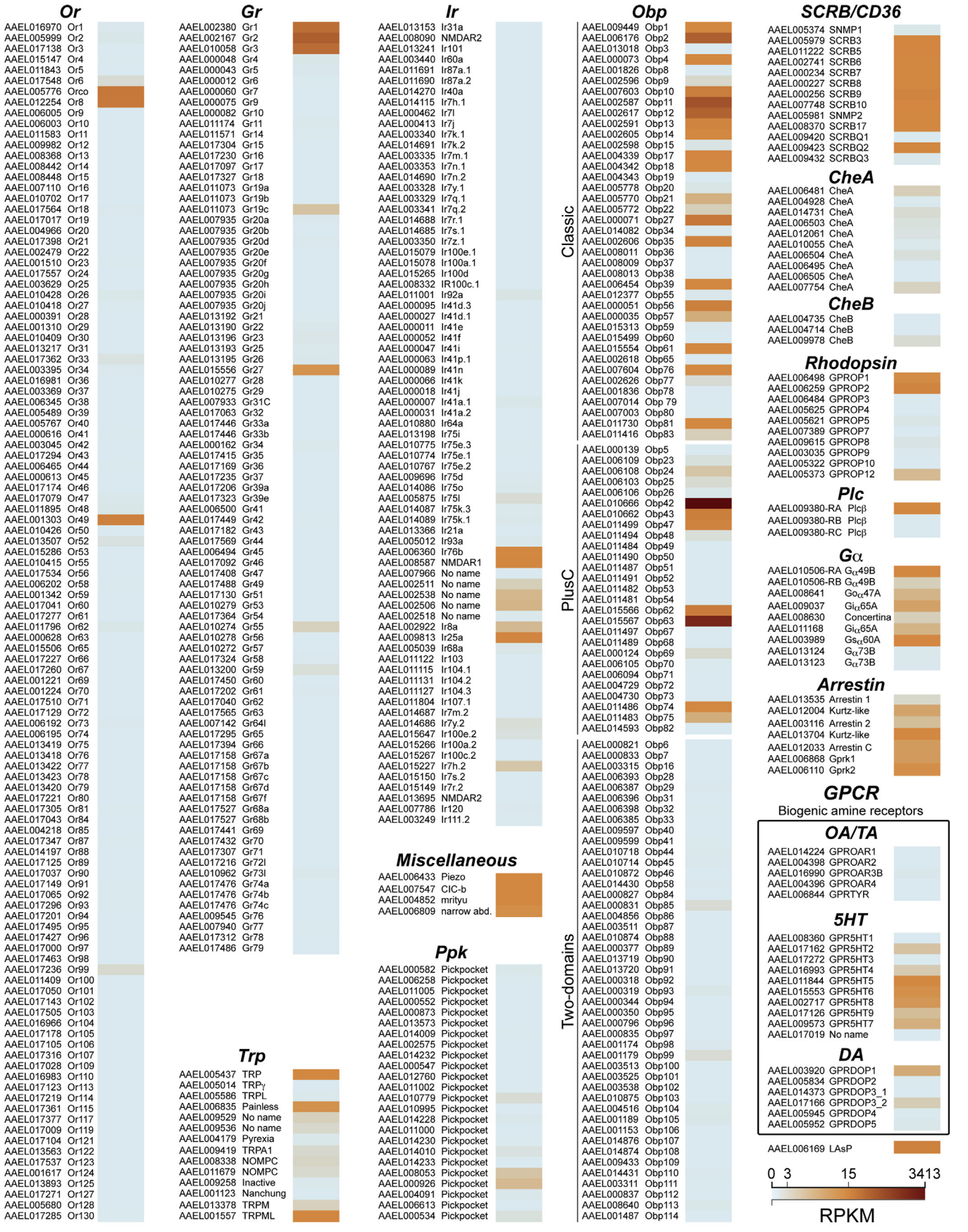


Fig. 3. Expression profile of the palp sensory transcriptome. The heat map shows the gene expression levels (in RPKM) of 503 genes putatively involved in olfaction, taste, mechanosensation, thermosensation, signal transduction and neuromodulation.

while phospholipases and G-proteins may function downstream of ligand activation of chemoreceptor proteins and upstream of *Trp* channels in sensory cells (Wicher et al., 2008; Badsha et al., 2012). We found that *Ae. aegypti* homologs of *Plcβ* (27 RPKM), *Trp* (23 RPKM) and *Gα49B* (36 RPKM) are expressed in the maxillary palp at similar levels (Fig. 3), which suggests that these three genes may function downstream of *Gr1* and *Gr3* to confer normal CO_2 responses (Fig. 1D). Three additional *Gr* genes were expressed at levels above 3 including the *DmelGr28* homolog *Gr19c* (6 RPKM) and the mosquito-specific *Gr27* (14 RPKM) and *Gr55* (4 RPKM) (Fig. 3). The function for these genes is unclear considering the maxillary palps of *Ae. aegypti* do not harbor conventional uniporous taste sensilla.

Ninety-five *Irs* have been identified in *Ae. aegypti* (Croset et al., 2010) of which 75 have received an accession number in NCBI (Fig. 3); *Ae. aegypti* shares 37 *Ir* orthologs with *D. melanogaster*. By comparison, only one clear OR ortholog (*Orco*) is shared between these two species (Bohbot et al., 2007). Among the *Ir* orthologs, the co-receptors *Ir8a* (7 RPKM), *Ir25a* (97 RPKM) and *Ir76b* (15 RPKM) were expressed in the maxillary palp (Fig. 3). Based on studies in *D. melanogaster* (Benton et al., 2009), it is likely that these co-receptors are expressed with one or several of the 5 additional *Ae. aegypti*-specific *Ir* genes (AAEL008587, AAEL002511, AAEL2538, AAEL002506 and Ir7h.2) also present in the maxillary palps (Fig. 3). The presence of *Irs* in the palp of mosquitoes has previously been reported in *An. gambiae* (17). Amongst the previously annotated *Irs* (Croset et al., 2010), AAEL008587 (Fig. 3) is the closest homolog to the *D. melanogaster* NMDA receptor 1 (NMDAR1) with 76% protein sequence identity (Supplemental Fig. 2). *D. melanogaster Nmdar1* has been implicated along with other factors such as pickpocket genes and *Trp* channels in larval mechanosensation (Fig. 1D) (Tsubouchi et al., 2012). The presence of expressed *Irs* in the maxillary palp, which are not known to co-express with *Ors* (37), suggests that they express in other neuronal contexts: (i) in the CO_2^- and human odor-sensitive "A" neuron, (ii) in other olfactory receptor neurons (ORNs) innervating capitate sensilla basiconica, or (iii) in unreported chemosensory sensilla. In *D. melanogaster*, *Ir* genes are not generally expressed in the sensilla basiconica and trichoidea, but are expressed in sensilla coeloconica, where in most cases ORco is absent (Benton et al., 2009). If these anatomical associations are conserved in mosquitoes, *Irs* in the *Ae. aegypti* maxillary palps may not be expressed in the capitate sensilla basiconica. To date, there have been no reports of other chemosensory organs on the maxillary palp surface (McIver, 1972).

Sensory Neuron Membrane Proteins (*Snmps*) belong to the Scavenger Receptor Type B gene family (*SCRB/CD36*) (Vogt and Riddiford, 1981; Rogers et al., 1997, 2001), which have functions as ligand-binding receptor molecules involved with cell: cell communications and ligand internalization (Vogt et al., 2009; Vogt, 2003). We confirmed the identity of thirteen putative *Ae. aegypti* SCRB genes including putative chemosensory genes *Snmp1 and Snmp2* by BLAST analysis using known *D. melanogaster* SCRB genes. No additional SCRB genes were uncovered (data not shown). *Snmp1* is required for the detection of the pheromone cis-vaccenyl acetate in *D. melanogaster* (Benton et al., 2007; Jin et al., 2008). Notably, *Snmp1* showed no expression in the maxillary palps of *Ae. aegypti* whereas its closest relative, *Snmp2,* was expressed (Fig. 3). Nine other SCRBs are expressed in this tissue. The functional significance of these proteins in the maxillary palps is unknown; however, they are well conserved among most insect species (Vogt et al., 2009) and may have non-chemosensory roles (Vogt, 2003).

In addition to *Ors*, *Grs*, *Irs* and *Snmps*, soluble proteins may affect chemosensation. The *Ae. aegypti* genome contains 111 *Obp* genes subdivided into three subfamilies that have been defined based on a bioinformatics approach (Zhou et al., 2008; Manoharan et al., 2013) (Fig. 3). OBPs are small soluble proteins secreted into the sensillum lymph and thought to participate in the detection of small volatile compounds by selective recognition and transportation to cell surface receptors (Vogt et al., 1999; Vogt, 2003). Classic OBPs carry a conserved motif of 6 cysteine residues, Plus-C OBPs harbor additional cysteine residues, and two-domain OBPs are the largest OBP subfamily (Zhou et al., 2008; Manoharan et al., 2013). There was no notable expression of two-Domain *Obps* in the female maxillary palps (Fig. 3). However, the largest number of *Obps* belonged to the *Classic* (56%) and to the *Plus-C* (33%) groups with expression levels above 3 RPKM (Fig. 3). Expression bias for *Obp1* and *Obp2* in the olfactory tissues of *Ae. aegypti* have previously been reported using quantitative Real-Time PCR (Sengul and Tu, 2010). The expression levels of *Obp1* and *Obp2* were 2.5 and 200-fold higher in the palp and proboscis than in the legs of 5-day-old adult females (Sparks et al., 2013); these levels are consistent with our data. *An. gambiae Obp1*, which is expressed in the antennae and maxillary palps, has been reported to specifically bind to the oviposition attractant indole (Biessmann et al., 2010). However, the indole receptor (OR2) (Bohbot et al., 2010), expressed in adult and larval antenna, is absent from the maxillary palps (Bohbot et al., 2007), making the function of *Obp1* in the context of the maxillary palps unclear. Among the highest expressed genes in our survey were *Obp63* (2373 RPKM) and *Obp42* (3413 RPKM) (Fig. 3); functional information regarding these genes and others of this family is lacking. In all, 22 Classic *Obps* are expressed in the maxillary palp, which possesses 3 chemosensory neuron types and recognizes only a handful of chemical cues. Therefore, we posit that these OBPs are likely not functioning as one to one carriers of chemical information to specific chemoreceptors as may be the case for *D. melanogaster* reception of the pheromone cis-vaccenyl acetate (Laughlin et al., 2008; Gomez-Diaz et al., 2013). Rather we propose that OBPs, as an ensemble, define which and how often certain low solubility odorants may reach the chemoreceptor interface.

CheA and *CheB* genes encode small soluble proteins unrelated to *Obps*. Expressed in contact chemoreceptor sensilla (Xu et al., 2002), they are involved with the detection of cuticular hydrocarbons (Park et al., 2006) required for normal courtship in *D. melanogaster*. *CheB* genes may interact with Degenerin/Epithelial Na^+ channels to detect these contact pheromones (Ben-Shahar et al., 2010). We detected very low expression levels of a few homologs of these genes expressed in the *Ae. aegypti* maxillary palps (Fig. 3). Since the palp is not known to engage in physical contact, it is unclear what role, if any, these genes have in the maxillary palps of *Ae aegypti*.

The presence of 6 *Grs*, including those related to insect CO_2 receptors, three *Ors* and several *Irs* confirms the role of the maxillary palps as olfactory appendages. These genes may be involved in the capitate sensilla basiconica ORN "A" response to human skin odors (Tauxe et al., 2013). Furthermore, the presence of this limited number of chemoreceptors (*Ors*, *Grs* and *Irs*) contrasts the plethora of expressed *Obps* and highlights a potentially useful model towards more broadly defining *Obp* function.

3.4. Thermosensation

Accurate temperature sensing is crucial for mosquito survival. While mosquitoes and other insects must avoid excessively cold or hot temperatures, mosquitoes are also attracted to the narrow temperature range of their vertebrate hosts (Howlett, 1910). On the antennae, the sensilla coeloconica detect high temperature (Davis and Sokolove, 1975), whereas thermoreceptors on the maxillary palps have not been described. *D. melanogaster* possesses at least three distinct molecular mechanisms to detect temperature changes (Fig. 1D). *TrpA1*, *Painless, Pyrexia* and *Plcβ* are involved in

noxious heat detection (Fowler and Montell, 2013). *Rhodopsin1* (*Rh1*), *Gα49B* (also known as *Gq*) and *Plcβ* while functioning in light detection, also allow *D. melanogaster* to discriminate between 18 °C and 24 °C (Shen et al., 2011). Finally, Gustatory receptor 28 (*Gr28*) has been implicated in the detection of rapid temperature changes in adult flies (Ni et al., 2013). Gene homologs expressed in the maxillary palp of *Ae. aegypti* suggest similar thermosensory capabilities.

The Transient Receptor Potential gene family is comprised of 13 genes belonging to 7 subfamilies (Supplemental Fig. 3) (Montell et al., 2002; Fowler and Montell, 2013), several of which are involved in temperature sensing. *TrpA1* (3 RPKM), *Pyrexia* (1 RPKM) and two *Trp* genes (3-4 RPKM) related to *Painless* (AAEL009529 & AAEL009536) were expressed just above background levels (Fig. 3). With such low level of expression, it is difficult to speculate whether these genes are indeed involved with thermosensation in the maxillary palp. Determining cell localization of these genes might provide insight into their function in this tissue. The relatively higher abundance of *Painless* (14 RPKM) suggests that the maxillary palp possesses nociceptors (Tracey et al., 2003). Whether *Painless* is involved in detection of mechanosensory or thermosensory stimuli is unknown. However, the presence of both *Piezo* (32 RPKM) and *Painless* in the maxillary palps suggests a mechanosensory role for the latter (see discussion below). The minute expression of *TrpA1* suggests that a few cells might be able to act as warmth sensors as shown in the *D. melanogaster* "anterior cell neurons" (Hamada et al., 2008). Noxious heat sensing is most likely carried out by the antennae as has been reported in *Ae. aegypti* (Davis and Sokolove, 1975) and *An. gambiae* (Wang et al., 2009).

Of the ten *Ae. aegypti* visual genes (*Rhodopsins*) (Nene et al., 2007), *Gprop12* (7 RPKM), *Gprop*1 (14 RPKM) and *Gprop*2 (116 RPKM) are expressed in the maxillary palps (Fig. 2). Compared to *D. melanogaster* rhodopsins, *Gprop*2 is homologous to *Rhodopsin*6 (60% amino-acid identity), *Rhodopsin*2 (56% amino-acid identity) and *Rhodopsin*1 (59% amino-acid identity) (Nene et al., 2007). *Rhodopsin*6 (Huber et al., 1997) is a green sensitive pigment expressed in the visual system of larval (Helfrich-Forster et al., 2002) and adult *D. melanogaster* (Yasuyama and Meinertzhagen, 1999). *Rhodopsin*2 (Cowman et al., 1986) is a violet-sensitive-pigment specifically expressed in the ocelli of the adult fly (Pollock and Benzer, 1988). *Rhodopsin*1 is a blue-sensitive photoreceptor expressed in the adult eye of *D. melanogaster* (O'Tousa et al., 1985; Zuker et al., 1985). In the fly visual system, TRP, TRPL, TRPγ (Montell, 2005), *Plcβ* (Shen et al., 2011) and Arrestin1/Arrestin2 (Montell, 2012) contribute to phototransduction. We detected *Trp* (23 RPKM) and *Plcβ* (27 RPKM) in the *Ae. aegypti* palp (Fig. 3), which excludes visual detection but is consistent with thermosensation and/or CO_2 detection (Fig. 1D). The *Ae. aegypti* homolog to *D. melanogaster Gα49B* (Supplemental Fig. 4), which is involved in phototransduction, is also significantly expressed (36 RPKM) in the mosquito maxillary palps (Fig. 2). We found minimal expression of the *D. melanogaster Arrestin1*/*2* homologs (Fig. 2). It is surprising that *Rhodopsins* are expressed in the maxillary palp of *Ae. aegypti* and the antennae of *An. gambiae* (Rinker et al., 2013), as both appendages lack visual sensory organs. Based on *D. melanogaster* experimentation, lack of visual organs on the palp, specific gene combination and phylogenetic analyses, we posit that *Gprop1* and *Gprop2* in combination with *Gα49B* and *Plcβ* are involved in heat sensing in adult mosquitoes (Fig. 1D).

The *D. melanogaster* paralogs *Gr28a* and *Gr28b* express in gustatory neurons in the adult and larval taste organs, as well as in non-chemosensory neurons in the central nervous system (Thorne and Amrein, 2008). Despite their designation as gustatory receptors, *Gr28b* mediates light avoidance behavior in fly larvae (Xiang et al., 2010) and splice variant *Gr28b(D)* acts as a thermosensor in adult flies (Ni et al., 2013). Peptide sequence alignment and phylogenetic analyses support an orthologous relationship between *Ae. aegypti* GR19c and *D. melanogaster* GR28 (Ni et al., 2013). We detected moderate *Ae. aegypti Gr19c* expression in the maxillary palp (Fig. 3). Like *Gr28a*, *Gr19* is differentially spliced into multiple isoforms whose functions remain unknown (Kent et al., 2008), although *Ae. aegypti Gr19c* is also expressed in the labella and tarsi (Sparks et al., 2013).

A temperature sensitive gustatory neuron expressing *TrpA1* has been identified in the moth, *Manduca sexta* (Afroz et al., 2013) and *TrpA1* has been found in porous sensilla coeloconica on the antennae of *Ae. aegypti* (Wang et al., 2009). Neurons within the capitate sensilla basiconica on the palp of *Ae. aegypti* may function as multimodal receptors integrating both olfactory and thermosensory information. Alternatively, thermosensitive *Rhodopsins*, *Trps* and a unique *Gr* may be expressed in neurons not located in any sensory organs. Cytolocalization of these genes in the maxillary palps of *Ae. aegypti* would help to resolve these questions and provide information as to their usefulness as targets for future repellent or attractant chemical development. Insects use at least three strategies to detect temperature changes and all three mechanisms are possible in the maxillary palps including thermo-*Trps*, a thermo-*Gr* and thermo-*Rhodopsins*.

3.5. Mechanosensation

Mechanosensation is the modality dedicated to the detection of humidity, sound, gravity, light and noxious touch (Fowler and Montell, 2013). *D. melanogaster* employs two different mechanotransduction mechanisms and at least ten genes to distinguish between noxious and gentle touch. Noxious touch is mediated by *Painless*, *Piezo* and *Ppk1* (Tracey et al., 2003; Kim et al., 2012; Johnson and Carder, 2012). *No Mechanoreceptor Potential C* (*nompC*), *Ppk2*, *chloride channel-b*, *narrow abdomen*, *mrityu*, *Nmdar1* and *Nmdar2* are required for mild touch detection (Walker et al., 2000; Liang et al., 2011; Yan et al., 2013). In the *Ae. aegypti* genome, we have identified homologs to the *D. melanogaster* genes involved in touch. *Painless* and *Ppk1* contribute to high-threshold sensitivity to mechanical stimuli in *D. melanogaster* larval class IV multiple-dendritic nociceptors (Johnson and Carder, 2012), which is consistent with the maxillary palp sensing noxious mechanical stimuli (Fig. 1D). We did not find the putative ortholog (AAEL013573) of *D. melanogaster Ppk1* in the *Ae. aegypti* maxillary palp. Instead, we found two *Ppk1* homologs in the *Ae. aegypti* maxillary palps: AAEL008053 (6 RPKM) and AAEL000926 (7 RPKM) (Fig. 3), which are also homologous to the *D. melanogaster ripped pocket* (*Rpk*/*Ppk2*) and *Ppk26* genes, belonging to the Ppk subfamily V (Zelle et al., 2013) (Supplemental Fig. 5). *Ppk26*, *Rpk* and *Ppk1* are expressed in different types of larval multidendritic neurons that are mechanosensitive (Zhong et al., 2010; Tsubouchi et al., 2012; Zelle et al., 2013). The *D. melanogaster* ion channel Piezo (*DmelPiezo*) is required for the detection of noxious touch (Kim et al., 2012). *DmelPiezo* is expressed in a variety of sensory organs in larvae and adult flies including sensilla campaniformia and chaetica (Kim et al., 2012). *DmelPiezo* was recently identified in *Ppk*-positive neurons and drives mechanically activated currents (Kim et al., 2012). We found only one homolog of *DmelPiezo* in the *Ae. aegypti* transcriptome (AAEL006433, 32 RPKM) (Fig. 3). The expression of *Painless*, *Piezo* and two homologs of *Ppk2* in the mosquito palp (Fig. 3) suggests that the palp is sensitive to noxious touch (Fig. 1D). The presence of *chloride channel-b* (24 RPKM), *mrityu* (18 RPKM), *na* (14 RPKM), *Nmdar1* (47 RPKM) and two Class V *Ppk* homologs suggests a role in mild touch detection for this group of genes in *Ae. aegypti* (Fig. 1D). However, the lack of expression of an *Nmdar2* and the low expression of *nompC* (2-3

RPKM) homologs is puzzling since these genes are required for this sensory modality in *D. melanogaster*. Expression of *DmelGR28a*, *DmelGr28b.b*, *DmelGr28b.c* and *DmelGr28b.d* has been associated with proprioceptive sensilla campaniformia on the wings of *D. melanogaster* (Thorne and Amrein, 2008). The *Ae. aegypti* maxillary palps express most of the genes involved with gentle and noxious touch in *D. melanogaster*. The relatively low expression of these mechanosensory genes, compared to *Ors* or *Grs*, indicates that these genes may be expressed in a restricted number of mechanosensory structures such as the sensilla chaetica or some other mechanosensory organ (Fig. 1C). Consistent with this idea is the observation that *DmelPiezo* is detected in adult sensilla chaetica and sensilla campaniformia (Kim et al., 2012). Sensilla campaniformia are external stretch receptors (proprioception) innervated by a single neuron that senses surrounding cuticular deformations (Fig. 1C). The discovery of a previously unreported sensory structure on the maxillary palps of *Ae. aegypti* raises the possibility that other structures have yet to be identified on this sensory appendage.

We did not find any genes associated with hygrosensation or sound detection. Humidity is an important signal for blood-feeding insects, although the cellular and molecular mechanisms by which sensory neurons are stimulated by changes in humidity are unclear (Fowler and Montell, 2013). Sensilla basiconica on the maxillary palps of the tsetse fly *Glossina morsitans* are sensitive to changes in relative humidity (Chappuis et al., 2013), and behavioral responses of *Ae. aegypti* to humidity have been reported (Eiras and Jepson, 1994). Since capitate sensilla basiconica on the maxillary palps of both *Ae. aegypti* and *Glossina* share common structural and neuronal organization, it would be interesting to investigate the physiological responses of these neurons to changes in humidity and heat in *Ae. aegypti*. In adult *D. melanogaster*, several gene families have been associated with water and humidity detection. The antennal-expressed *Trp* channel genes *waterwitch* and *nanchung* are required to detect dry and moist air, respectively (Liu et al., 2007). *D. melanogaster Ppk28*, expressed in taste sensilla on the labellum, legs and wing margins, is also involved in water detection (Chen et al., 2010). We did not detect *nanchung*, *waterwitch* or *Ppk28* homologs in the maxillary palps of *Ae. aegypti* (Fig. 2), which is consistent with previous observation (Roth, 1952; Bar-zeev, 1960) that the maxillary palps do not detect humidity. Sound detection in *D. melanogaster* requires *Nanchung* (*Nan*) and *inactive* (*Iav*), which are two TRPV channels (Fig. 1D) (Gong et al., 2004; Zhang et al., 2013). *Ae. aegypti* homologs of these genes were not found in the maxillary palp (Fig. 3). Gravity detection in the chordotonal neurons of adult flies is generally associated with *Painless* and *Pyrexia* (Sun et al., 2009), both members of the TRPA subfamily (Supplemental Fig. 3). The lack of *pyrexia* in this study suggests that Painless is not utilized in the context of gravity sensation (Fig. 1D).

3.6. Neuromodulation and regulation of neural signaling

We surveyed the expression of 21 biogenic amine receptors reported in the *Ae. aegypti* genome (Nene et al., 2007). Octopamine and tyramine receptors are not expressed in maxillary palp of 6-day old adult females (Fig. 2), and there was little expression of the dopamine D1 receptor homolog (Nene et al., 2007; Meyer et al., 2012) (Fig. 3). By contrast, 7 of the 10 identified serotonin receptor transcripts were present in this tissue (Fig. 3). The serotonergic systems of two Dipterans, namely the blow fly *Phormia regina* and the mosquito *Ae. triseriatus*, have been shown to affect feeding (Long and Murdock, 1983; Novak and Rowley, 1994). Serotonin has been localized in the central (including the antennal lobes) and peripheral nervous systems (Siju et al., 2008) and in the salivary glands (Novak et al., 1995) of *Ae. aegypti*. Serotonin-immunoreactive fibers have been described originating from the CNS and extending through the suboesophageal ganglion to innervate the maxillary palp where they likely modulate neuronal activity through the serotoninergic receptors (Siju et al., 2008). These fibers form more extensive ramifications in the fourth palp segment where capitate sensilla basiconica are located. Our study confirms that serotonin and to a lesser extent dopamine receptors are expressed in some cell types associated with the maxillary palps and suggests a possible role of these receptors in modulating sensory activity in the maxillary palps.

The *Arrestin* family is a small group of genes used by both vertebrates and insects to regulate cellular signaling pathways (Gurevich and Gurevich, 2006). We identified putative *Ae. aegypti* orthologs for all the *D. melanogaster Arrestin* genes (Supplemental Fig. 6). In the ommatidia of insects, Arrestin1 and Arrestin2 quench the photo response by interacting with Rhodopsins. The relatively low levels of *Arrestin1* (3 RPKM) and *Arrestin2* (7 RPKM) expression in the maxillary palp are inconsistent with a role in phototransduction. In flies, *G-protein receptor kinases* (*Gprks*) phosphorylate ligand-activated GPCRs while *Kurtz* promotes the internalization of the receptors. We found moderate expression of *Kurtz*- (27 RPKM) and *Gprk*- (12–14 RPKM) like genes in the maxillary palps (Fig. 3). What receptors, if any, do these proteins regulate in the maxillary palps of adult mosquitoes? Arrestins and Gprks are ubiquitous regulators of GPCRs. In the maxillary palp these genes may regulate GPCRs including the serotonin receptors or the Rhodopsins. However, Gprks can also interact with a variety of non-GPCRs (Gurevich et al., 2012). *DmelKurtz* is involved with the development of the central and peripheral nervous system (Roman et al., 2000). In the adult fly, *Kurtz* expresses in ORNs where it regulates olfactory sensitivity (Ge et al., 2006). The expression of *Kurtz*-like and *Gprks* genes in the maxillary palps of *Ae. aegypti* and in the fly ORNs suggests that they may interact with ORs and G-proteins (Fig. 1D). $G\alpha_s$ in particular has been shown to regulate olfactory signal transduction in fly ORNs but not in CO_2 sensing neurons (Deng et al., 2011). The highest expression for a G-protein in the *Ae. aegypti* maxillary palp was $G\alpha_s$ (42 RPKM), which is consistent with the expression level of this gene in the antennae of *An. gambiae* (Rutzler et al., 2006). The view that insect ORs form ligand-gated ion channels that are metabotropically modulated (Wicher, 2013) is consistent with this observation.

4. Conclusions

The goal of this study was to explore the genetic components involved with sensory acquisition and sensory neuromodulation in a relatively simple appendage. The maxillary palps of female *Ae. aegypti* perceive stimuli in close proximity to the host (Roth, 1951; Rahm, 1958; Tauxe et al., 2013) and vibrate during proboscis insertion and withdrawal when blood-feeding (McIver, 1972). Here, we have attempted to infer sensory gene networks involved in this behavior based on the transcript expression profile of the maxillary palps in combination with studies conducted in another Dipteran, *D. melanogaster*. We have identified genes involved in mechanosensation and uncovered candidate genes involved with thermosensation, a sensory modality that was identified in the maxillary palp in 1951 (Roth, 1951).

We confirm that the maxillary palps only express three *Ors*. The identification of three *Grs*, in addition to the previously reported CO_2 receptors, including a possible thermo-*Gr* is an interesting find as is the confirmation of olfactory *Irs* in this tissue. These receptors may be involved in the maxillary palp responses and resulting behavioral attraction to human skin odorants (Tauxe et al., 2013). The presence of relatively few chemoreceptor transcripts (*Ors*, *Grs*

and *Irs*) contrasts the plethora of expressed *Obps* and brings into question the role of these proteins in context of chemosensation. We propose that OBPs likely modulate the passage of low solubility odorants through the aqueous lumen to the chemoreceptor interface in a combinatorial fashion (Nardi et al., 2003), rather than forming strict associations with specific chemicals.

Mechanosensation in the palp seems to include responses to both mild and noxious stimuli based on *nompC* and *Painless* expression. Insects use at least three strategies to detect temperature changes and all three mechanisms in the maxillary palps of *Ae. aegypti* may be mediated by thermo-*Trps*, thermo-*Rhodopsins* and a thermo-*Gr*.

Six parallel molecular sensory pathways may be operating in the maxillary palp of female *Ae. aegypti* (Fig. 1D). Considering, that some of these mechanisms do not overlap in the same neurons, and that instances of multimodal neurons have been reported (Afroz et al., 2013; Kim et al., 2010), it is likely that the maxillary palp ORNs exhibit multimodal capabilities. Without cellular localization of these genes, it will be difficult to attribute these additional modalities to the sensory organs (sensillum chaetica, campaniformia and basiconica) of the maxillary palp and the sensory neurons they contain.

Neuromodulation of sensory signals in the maxillary palps appears to be exclusively mediated by the serotonergic pathway. Finally, we provide circumstantial evidence that regulation of olfactory signaling is carried out by Arrestins and G-proteins possibly coupling to ORs.

In this study, Illumina next-generation sequencing was applied to survey sensory genes in the maxillary palps of female *Ae. aegypti*. In addition to unraveling the genetic basis of sensory processing in this important appendage, our study provides potential molecular targets for disruption of mosquito behavior. Due to their unique neuronal location and function, ORs, GRs, IRs, TRPs, Rhodopsins and Ppks represent attractive targets for pharmacological intervention.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2014.02.007.

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