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The genetics of chemoreception in the labella and tarsi of *Aedes aegypti*

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ABSTRACT

The yellow-fever mosquito Aedes aegypti is a major vector of human diseases, such as dengue, yellow fever, chikungunya and West Nile viruses. Chemoreceptor organs on the labella and tarsi are involved in human host evaluation and thus serve as potential foci for the disruption of blood feeding behavior. In addition to host detection, these contact chemoreceptors mediate feeding, oviposition and conspecific recognition; however, the molecular landscape of chemoreception in these tissues remains mostly uncharacterized. Here we report the expression profile of all putative chemoreception genes in the labella and tarsi of both sexes of adult *Ae. aegypti* and discuss their possible roles in the physiology and behavior of this important disease vector.

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1. Introduction

Aedes aegypti (L.) (Diptera: Culicidae) mosquitoes contribute to the spread of dengue, yellow fever, chikungunya and West Nile viruses through blood meal-mediated viral transmission. The global health risks associated with this disease vector are increasing (Bhatt et al., 2013; Guzman et al., 2010; Barrett and Higgs, 2007), as these mosquitoes thrive in urban environments. With potential vaccines still in development (Wallace et al., 2013), bite prevention is essential to curb the spread of these deadly diseases.

Disruption of mosquito host-seeking and feeding behavior has been achieved through use of several repellent chemicals like DEET and Picaridin (Dickens and Bohbot, 2013); and while progress has been made towards understanding the molecular associations of these repellents, their mechanisms of action remains unclear. The repellency response appears to involve multiple chemoreception pathways. *Ae. aegypti* avoidance of volatile DEET is mediated by the Odorant Receptor gene family, a molecular pathway also involved in DEET perception in the dipteran relative *Drosophila melanogaster* (Ditzen et al., 2008; DeGennaro et al., 2013). Transgenic *Ae. aegypti* lacking this olfactory pathway will land on DEET treated human skin, but will not blood-feed after contact with DEET, suggesting that non-olfactory molecular pathways of the tarsi, labella or elsewhere are involved in DEET sensation and behavioral avoidance (DeGennaro et al., 2013). Recently, Ionotropic Receptors have also been shown to play a role in DEET avoidance in *D. melanogaster* (Kain et al., 2013), highlighting the need to investigate the role of all known insect chemoreception gene families in the avoidance of repellents by mosquitoes.

Interestingly, DEET avoidance by *D. melanogaster* in feeding assays requires at least three Gustatory Receptors (Lee et al., 2010). In *Ae. aegypti*, bitter sensing gustatory receptor neurons (GRNs) of the labella respond to DEET and other repellents in electrophysiological recordings (Sanford et al., 2013), but this response's effect on mosquito behavior is unknown. Thus, putative chemoreception genes expressing in the GRNs of the major gustatory appendages may serve as useful targets to develop novel deterrents.

Several classes of genes involved in chemoreception have been identified in insects: gustatory receptors (*Grs*) (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001; Robertson et al., 2003), odorant receptors (*Ors*) (Clyne et al., 1999; Vosshall et al., 1999; Robertson et al., 2003), ionotropic receptors (*Irs*) (Benton et al., 2009; Croset et al., 2010), odorant binding proteins (*Obps*) (Vogt and Riddiford, 1981; McKenna et al., 1994; Galindo and Smith, 2001), sensory neuron membrane proteins (*Snmps*) (Rogers et al., 1997; Vogt et al., 1999; Benton et al., 2007; Jin et al., 2008),

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pickpocket channels (*ppks*) or DEG/ENaCs (Liu et al., 2003), transient receptor potential channels (*Trps*) (Al-Anzi et al., 2006; Kwon et al., 2010; Kim et al., 2010; Kang et al., 2010) and CheA/Bs (Xu et al., 2002; Park et al., 2006). These genes are typically expressed in chemosensory neurons or support cells associated with peripheral hair-like sensory organs called sensilla. These chemosensory neurons discriminate between diverse chemicals, often informing mosquito behaviors such as host-seeking, feeding, oviposition and mating.

There are few comprehensive reports of chemoreception gene expression in specific appendages in dipteran species; confirmation of expression in *D. melanogaster* has been shown through non-quantitative visual representations using promoter-driven reporters or amplified PCR products, with these reports being organized by chemoreception gene family rather than global expression profile (Vosshall et al., 1999; Galindo and Smith, 2001; Couto et al., 2005; Benton et al., 2009; Isono and Morita, 2010). In *Ae. aegypti*, characterization of *Or* expression has been conducted non-quantitatively by PCR in all head appendages (Bohbot et al., 2007), and three *Grs* have been studied in the maxillary palps (Erdelyan et al., 2012; Bohbot et al., 2013). We previously reported the expression profile of *Grs* in the labella and tarsi of male and female *Ae. aegypti* (Sparks et al., 2013). Here we extend our survey to the expression of other putative chemoreception genes by RNA-seq in these tissues, thus expanding our knowledge of the appendage-specific molecular components potentially involved in chemical attraction and avoidance. Expressed putative chemoreception genes in male and female mosquitoes may be targeted in future experiments aimed at altering their behavior and ultimately disrupting transmission of harmful viruses. Furthermore, we assessed the significance of this expression for a small set of chemoreception genes by comparing RT-qPCR estimates of expression between female labella and female carcass tissue samples.

2. Materials and methods

2.1. Animal rearing

Ae. aegypti eggs (Orlando strain) were obtained from the Center for Medical and Veterinary Entomology, USDA, ARS in Gainesville, FL, USA. Larvae were reared at 25 °C (12-hL:12-hD) and fed with ground TetraMin® fish food. Unsexed pupae were hand-collected daily and transferred to plastic dishes (9 cm × 5.5 cm) inside small containment buckets, thus establishing 24-h age groups. Greater than 95% of adults emerged 2 days post-pupation, after which all remaining pupae were removed from containment buckets. Adult mosquitoes were fed with a 10% sucrose solution and maintained in an environmental chamber at 27 °C and 70% relative humidity under the same photoperiod as larvae. Tissues used in our studies were collected during the photophase from adult mosquitoes 6–7 days old.

2.2. RNA isolation and sequencing

For RNA sequencing, paired labella from 500 males or 500 females were carefully dissected to limit inclusion of other adjacent proboscis tissues. Samples from legs were comprised of pro-, meso-, or metathoracic tarsal segments of 400 males or 400 females. Dissected tissues were immediately stored on dry ice and mechanically ground in TRIzol® (Life Technologies, Carlsbad, CA, USA). Total RNA was isolated by RNeasy® Plus Mini Kit (Qiagen, Valencia, CA, USA), quantified on a Nanodrop ND-1000 spectrophotometer (Nano Drop Products, Wilmington, DE, USA), and sent to the Genomics Services Lab at the Hudson Alpha Institute for Biotechnology (Huntsville, AL). Messenger RNA isolation and subsequent cDNA synthesis were completed using NEBNext® reagents (NEB, Ipswich, MA, USA) and standard protocols with custom GSL adaptors. Complementary DNA libraries corresponding to distinct tissues were sequenced on an Illumina HiSeq2000 to generate 25 million 50 base pair, paired-end reads per sample.

2.3. Analysis of annotated and unannotated chemosensory genes

Reference genome and annotations for *Ae. aegypti* (AaegL1.3) were downloaded from VectorBase (http://aaegypti.vectorbase.org/GetData/Downloads/). Output Fastq Illumina files were mapped to the reference genome with TopHat (Trapnell et al., 2009). The unambiguous sequence alignment files were uploaded into the Avadis NGS software (Strand Scientific Intelligence, CA, USA), where quantification and normalization were performed. Prior to quantification using the 'Deseq' normalization method, the read list was filtered to remove duplicate, single-end, mate-filtered, mate-missing, one-mate flip, both-mate flip, and unaligned reads. Read quality metric values were as follows: Quality threshold ≥30, N's allowed in read ≤0, Alignment score threshold ≥95, Mapping quality threshold ≥40. Transcript expression levels for all genes are reported in values of Reads Per Kilobase per Million reads mapped (RPKM). RPKM values represent a quantitative measure of the number of corresponding 50 bp sequence reads (sequenced in both directions) for a given gene. We assigned no specific RPKM threshold for functional expression vs. background "noise."

2.4. Quantitative RT-PCR

Thirteen chemosensory genes and one housekeeping gene were selected for qPCR analysis to evaluate gene expression over a dynamic range, both in copy number and presumed chemosensory gene function. Primer pairs were designed for each target gene to amplify a specific 100–180 base pair PCR product (Primer-BLAST Primer Designing tool, NCBI). At least one primer per set spans an exon boundary to exclude non-specific gDNA amplification.

Statistically supported RT-qPCR validation of RNA sequencing of labella and all tarsal types of both sexes was previously reported in Sparks et al. (2013). Here we dissected 150 paired female labella and 10 female carcasses (thorax, halteres and abdomen) for RNA extraction, repeating each tissue once. Total RNA was isolated from all frozen tissue samples as previously described. cDNA was synthesized using Superscript® III First-Strand Synthesis Supermix for qRT-PCR (Life Technologies, Carlsbad, CA, USA). PCR products were directly sequenced to confirm amplicon identity (data not shown) (Macrogen, Rockville, MD, USA). RT-qPCR was subsequently performed on each target gene using KiCqStart® SYBR® Green qPCR ReadyMix™ iQ (Sigma–Aldrich, St. Louis, MO, USA) and an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA). All Ct values were calculated by Bio-Rad iQ5 Optical System Software (Bio-Rad, Hercules, California, USA). Reactions were performed in technical triplicate 20 μL volumes. Three-step cycles plus melt curves were used for each reaction, using an annealing temperature of 56 °C for all primers. Efficiencies for each primer set were calculated from the slope of the standard curve using the formula $E = 10^{(-1/\text{slope})}$ (Pfaffl, 2001; Rasmussen, 2001). Primer efficiencies are based on three 1:10 serial dilutions of cDNA template used in side-by-side technical triplicate reactions. Efficiencies are listed in Table S1.

Relative gene quantification was calculated as $E_{\text{target}}^{-(Ct[\text{target}]-Ct[\text{reference}])}$ for each target gene (14 total) and averaged for each replicate (6 total for each tissue sample, representing two biological replicates). *Ae. aegypti* housekeeping gene Lysosomal Aspartic Protease (Vectorbase ID: AAEL006169) was used to normalize Ct values between tissue types. 'Times-enrichment' was calculated as