Expansions of chemosensory gene orthologs among selected tsetse fly species and their expressions in *Glossina morsitans morsitans* tsetse fly

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Abstract

Tsetse fly exhibit species-specific olfactory uniqueness potentially underpinned by differences in their chemosensory protein repertoire. We assessed 1) expansions of chemosensory protein orthologs in *Glossina morsitans morsitans*, *Glossina pallidipes*, *Glossina austeni*, *Glossina palpalis gambiensis*, *Glossina fuscipes fuscipes* and *Glossina brevipalpis* tsetse fly species using Café analysis (to identify species-specific expansions) and 2) differential expressions of the orthologs and associated proteins in male *G. m. morsitans* antennae and head tissues using RNA-Seq approaches (to establish associated functional molecular pathways). We established accelerated and significant (P<0.05, λ = 2.60452e-7) expansions of gene families in *G. m. morsitans* Odorant receptor (Or)71a, Or46a, Ir75a,d, Ionotropic receptor (Ir) 31a, Ir84a, Ir64a and Odorant binding protein (Obp) 83a-b, *G. pallidipes* Or67a,c, Or46a, Or71a, Or68a, Or85b-c,f and Obp73a, *G. f. fuscipes* Ir21a, Gustatory receptor (Gr) 21a and Gr63a, *G. p. gambiensis* clumsy, Ir25a and Ir8a, and *G. brevipalpis* Ir68a and missing orthologs in each tsetse fly species. Most abundantly expressed transcripts in male *G. m. morsitans* included specific Or (Orco, Or56a, 65a-c, Or47b, Or67b, GMY012254, GMY009475, and GMY006265), Gr (Gr21a, Gr63a, GMY013297 and GMY013298), Ir (Ir8a, Ir25a and Ir41a) and Obp (Obp19a, lish, Obp28a, Obp83a-b Obp44a, GMY012275 and GMY013254) orthologs. Most enriched biological processes in the head were associated with vision, muscle activity and neuropeptide regulations, amino acid/nucleotide metabolism and circulatory system processes. Antennal enrichments (>90% of chemosensory transcripts) included clium-associated mechanoreceptors, chemo-sensation, neuronal controlled growth/differentiation and regeneration/responses to stress. The expanded and tsetse fly species specific orthologs includes those associated
with known tsetse fly responsive ligands (4-methyl phenol, 4-propyl phenol, acetic acid, butanol and carbon dioxide) and potential tsetse fly species-specific responsive ligands (2-oxopentanoic acid, phenylacetaldehyde, hydroxycinnamic acid, 2-heptanone, caffeine, geosmin, DEET and (cVA) pheromone). Some of the orthologs can potentially modulate several tsetse fly species-specific behavioral (male-male courtship, hunger/host seeking, cool avoidance, hygrosensory and feeding) phenotypes. The putative tsetse fly specific chemosensory gene orthologs and their respective ligands provide candidate gene targets and kairomones for respective downstream functional genomic and field evaluations that can effectively expand toolbox of species-specific tsetse fly attractants, repellents and other tsetse fly behavioral modulators.

Author summary

Tsetse flies are insect vectors of sleeping sickness in humans and nagana in livestock in sub-Sahara Africa. Tsetse flies identify their hosts (preferred and non-preferred) by detecting and processing odor cues emitted by the hosts in their environment. Tsetse flies use chemosensory proteins and associated pathways in their antennae to identify these cues. In this study, we identified expansions of these chemosensory protein in six tsetse fly species (Glossina morsitans morsitans, Glossina pallidipes, Glossina austeni, Glossina palpalis gambiensis, Glossina fuscipes fuscipes and Glossina brevipalpis) with different known hosts. We also identified potential ligands to these proteins based on fruit fly (Drosophila melanogaster) orthologs. With G. m. morsitans as an example, we identified the proteins and associated molecular pathways preferentially expressed in tsetse fly antennae. These proteins may be responsible for the tsetse fly species-specific host discrimination, with the ligands eliciting species-specific behavioral responses in the flies. The expressed orthologs may be functionally important in odor detection in tsetse fly and lay down useful groundwork for downstream functional genomics R&D for more effective tsetse fly species-specific odor attractants and repellents for routine tsetse fly control operations.

Introduction

Human African Trypanosomiasis (HAT) constitutes one of the most neglected tropical diseases (NTDs) with devastating health and economic consequences in sub-Sahara Africa [1,2]. On the other hand, African Animal Trypanosomiasis (AAT) is rampant in livestock inhabiting tsetse-infested areas throughout the continent. The AAT cause death of about three million cattle each year [3], and in terms of agricultural Gross Domestic Product (GDP), loss of about US$ 4.75 billion per year [3]. The HAT and AAT causative trypanosomes are transmitted by different groups of tsetse species. Tsetse control is considered an effective approach and constitutes the corner stone in trypanosomiasis suppression [4,5]. Tsetse fly species belong to Glossina genus and are generally restricted to sub-Saharan Africa. Twenty-three species and eight sub-species of tsetse flies are recognized [6,7]. These species are divided into Morsitans, Palpalis and Fusca clade sub-genera, described by respective savanna, riverine/lacustrine and forest ecological niches they occupy. The Morsitans group consists of five species that include Glossina morsitans morsitans and Glossina pallidipes restricted to savannah grassland and Glossina austeni occupying coastal woodlands [8]. This group is adapted to drier habitats than Palpalis and Fusca [9] and preferentially feeds on livestock and wildlife. They are thus important
vectors of African Animal Trypanosomiasis (AAT) also known as nagana. On the other hand, Palpalis group consists of five species, including Glossina palpalis gambiensis and Glossina fuscipes fuscipes in West, Central and East Africa. These species are predominant vectors of Human African Trypanosomosis (HAT), also known as sleeping sickness, despite their preferential predilection to feeding on reptiles and ungulates. Fusca group consist of 13 species largely inhabiting damp evergreen forests of West Africa (except Glossina brevipalpis) and are mainly associated with livestock. Glossina brevipalpis is of limited medical and agricultural significance and occurs discontinuously in other parts of sub-Saharan Africa [6].

These tsetse fly species exhibit different olfactory uniqueness, which partly accounts for their gradation of preferences for their particular hosts. This olfactory uniqueness (and visual responses) has been exploited in designing effective tsetse fly bait technologies that consist of synthetic blends of attractants and repellents that mimic those of their natural hosts and non-hosts respectively [10–13]. These technologies are especially applicable for G. m. morsitans and G. pallidipes but not G. austeni (among savanna species) [14] and palpalis group. For example, G. pallidipes, G. m. morsitans and to some extent G. brevipalpis are attracted to traps baited with POCA (3-n-propylphenol, 1-octen-3-ol, 4-cresol and acetone) and to which G. austeni poorly responds [15–17]. Molecular bases of these natural differential responses are poorly understood but may be underpinned by differences in their chemosensory apparatus. The chemosensory apparatus facilitate reception of odorants and tastants, and consist of Odorant-binding proteins (Obps), Odorant-degrading enzymes (Odes), Odorant receptors (Ors), Ionotropic receptors (Irs), Gustatory receptors (Grs), Chemosensory proteins (Csps), Sensory neuron membrane proteins (Snmps) and CD36-like pheromone sensors [18–24]. These chemosensory proteins mediate decoding of ecological odors and odorant specific behavioral responses in insect hosts. These responses include seeking for hosts, location of oviposition sites, searching for mates, and detecting and escaping from potential predators. The Obp transport pheromone molecules and general odorants to Ors [25]. The Ors are odorant-gated ion channels composed of an odorant-binding subunit and olfactory co-receptor Orco [26,27]. The Irs have higher specificity to volatiles than Ors, detecting specific variety of odors, such as acids, aldehydes, amines and humidity [20,28]. The Ir25a and Ir8a are putative conserved Ir co-receptors [23]. The Grs discern odor tastes and contact pheromones [29]. Only two Snmp subfamilies (Snmp 1 and Snmp 2) have been identified in insects, where Snmp1 is expressed in pheromone-sensitive Olfactory Receptor Neurons (ORNs) while Snmp 2 is expressed in supporting cells [30–32]. Some of these chemosensory proteins are present in non-canonical chemosensory organs, such as legs [33,34], wings [35,36] and pheromone glands [37], where only a subset of Irs are specifically expressed in D. melanogaster antennae [20]. Among tsetse flies, genomes of G. pallidipes, G. m. morsitans, G. austeni, G. p. gambiensis, G. f. fusipes and G. brevipalpis (representative of the different clades/sub-general) have been sequenced [38], and their respective chemosensory proteins annotated [39–41].

Here we report on 1) expansions of chemosensory protein orthologs in six tsetse fly species/subspecies (G. pallidipes, G. m. morsitans, G. austeni, G. p. gambiensis, G. f. fusipes and G. brevipalpis) to identify species-specific expansions and 2) differential expressions of these and associated proteins in antennae and head tissues G. m. morsitans to establish probable functional pathways influencing host seeking behaviors in this specie.

Materials and methods

Differential expansions of D. melanogaster chemosensory gene orthologs among tsetse flies

We obtained complete D. melanogaster gene set release 79 (Drosophila_melanogaster. BDGP6. pep.all.fa) from Ensembl project [42] in fasta format. We then isolated D. melanogaster
chemosensory genes from the gene set by searching and retrieving flybase [43] chemosensory gene IDs in the gene set using “Odorant receptor”, “Gustatory receptor”, “Ionotropic receptor”, “Odorant-binding protein”, “Sensory neuron membrane protein” and “Glutamate receptor” Linux bash regular expressions. For Csp orthologs, we extracted D. melanogaster IDs from Macharia et al., (2016) [40]. We separately obtained VectorBase Release VB-2019-02 homologs (gene trees) of disease vectors from VectorBase database [44] in OrthoXML formats. The gene trees were pre-computed by Gene Orthology/Paralogy prediction pipeline in VectorBase [44] that identified gene duplications within species and specification events. We probed the VectorBase homologs for ortholog groups (gene families) with the D. melanogaster chemonsensory genes (flybase IDs) to identify their respective tsetse flies (G. austeni, G. f. fuscipes, G. p. gambiensis, G. brevipalpis, G. pallidipes and G. m. morsitans) orthologs. We identified presence of the individual genes in each gene family (ortholog group) and species. Gene families with accelerated gene expansions were pre-computed through Computational Analysis of gene Family Evolution (CAFE) [45] in VectorBase [44]. We considered the VectorBase [44] pre-computed gene expansions/contractions reliable since they are 1) community reviewed and adopted and with stable ortholog IDs and 2) regularly updated (with new gene-sets and genomes). We also conducted Principal Component Analysis (PCA) in R using FactoMineR and Factoextra packages with species-specific gene counts as input data to establish relationship between the expanded/contracted chemosensory genes (Ors, Irs, Grs and Obps) and tsetse species.

**Transcriptional expression of D. melanogaster chemosensory gene orthologs in male G. m. morsitans**

We employed high throughput Illumina based RNA-Seq approach to establish expression profiles of the D. melanogaster chemosensory gene orthologs in male G. m. morsitans. We established expression levels of the orthologs in the antennae and in relation to the head libraries. We isolated and sequenced RNA from antennae or head tissues from colony reared G. m. morsitans as described previously [46]. Briefly, we fed teneral male G. m. morsitans (1–3 days old) on defibrinated bovine blood meal (their initial blood meal post-eclosion) (commercially supplied by Hemostat Laboratories, Dixon, CA, USA) to putatively prime their chemosensory system. We then extracted their antennae in two independent biological replicates (from 50 flies each) using liquid nitrogen-based method of Menuz et al. (2014) [47] 72 hrs post-feeding. We envisaged that the 72 hrs deprivation of blood meal (food) would biologically prime potential host seeking chemosensory apparatus in the flies and enhance RNA-seq detection of chemosensory gene expressions, specifically those associated with hunger/host seeking.

The G. m. morsitans show marked die1 changes in their biting activity in the field, with their peak activity in the morning and afternoon [48,49]. We thus snap froze individual tsetse flies in liquid nitrogen in the morning (09:30 hrs) and carefully hand-dissected their antennae from the head into 1.5 ml microfuge tubes kept cold in liquid nitrogen. We then isolated RNA by mechanically crushing the antennae with disposable RNaseq-free plastic pestles in TRIzol reagent (Invitrogen, Carlsbad, USA) following the manufacturer’s protocol. We removed traces of potential carry over DNA (that could potentially confound our RNA-Seq analysis) by digesting possible contaminating genomic DNAs (gDNA) in the total RNA using TURBO DNase (Ambion life technologies, TX, USA) following manufacturer’s instructions. We confirmed removal of the gDNA from total RNA by qualitative assessment of PCR amplicons from final RNA samples using tsetse fly specific beta-tubulin gene primers as documented in Bateta et al. (2017) [46]. We verified quality and integrity of RNA samples using Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) following manufacturer’s instructions. cDNA was
then generated from the RNA using Illumina TruSeq RNA Sample Preparation Kit (Illumina, Hayward, CA, USA) and the cDNA (75 bp single-end read) and sequenced on Illumina HiSeq 2500 at Yale University Center of Genome Analysis (YCGA), New Haven, CT, USA. We similarly prepared head transcriptomes from two independent biological replicates (50 flies each) from 72 hrs starved 40 days old males. We deposited all transcriptome sequences at the Sequence Read Archive (SRA) under study accession numbers PRJNA343267 and PRJNA343269 for the antennae and head libraries respectively.

Expression profiles of *D. melanogaster* chemosensory gene orthologs in male *G. m. morsitans* antennae and head libraries

We established quality of the reads in each individual transcriptome library using FastQC (Babraham Bioinformatics) software package (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). We then used the FastQC results to clean (trim) the reads using CLC genomic workbench version 10 software (CLC Bio, Aarhus, Denmark) through settings that permitted 1) removal of low quality sequences (limit = 0.05), 2) removal of ambiguous nucleotides (maximum 2 nucleotides allowed), 3) removal of terminal nucleotides (10 nucleotides from the 5’ end and 1 nucleotide from the 3’ end) and 4) removal of sequences on length (minimum length 15 nucleotides, maximum length 1000 nucleotides). We then mapped the cleaned reads on to *G. m. morsitans* transcripts gene-set version 1.9 from Vectorbase [44] using CLC genomic workbench version 10 software (CLC Bio, Aarhus, Denmark) thorough settings that permitted 1) mismatch cost of 2, 2) insertion/deletion cost of 3, 3) length fraction of 0.8, 4) similarity fraction of 0.8, 5) maximum number of reads per hit of 10, and 6) strand specificity set as both strands.

From the mappings, we established reads mapping per transcript and reads per kilobase of transcripts per Million mapped reads (RPKM), a normalized index of relative gene expression associated with each transcript (including chemosensory genes) in the gene-set for individual transcriptomes [50]. We then established differentially expressed transcripts between the antennae and the head transcriptomes by comparing the reads mapped in the genes sets from respective transcriptomes using edgeR software [51,52]. We considered transcripts validly differentially expressed if they had at least two-fold changes, p-value corrected False Detection Rate (FDR) < 0.05 and one Counts Per Million (CPM) coverage to mitigate against type I statistical errors. We then determined antennae or head enriched molecular processes using canonical Gene Set Enrichment Analysis (GSEA) using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt) [53]. Since WebGestalt database did not include tsetse flies, but *D. melanogaster* gene set, we obtained homologs of the entire *G. m. morsitans* gene-set in *D. melanogaster* through Basic Alignment Search Tool (BLAST) analysis of protein sequences (Blastp) [54] of the *G. m. morsitans* gene-set against those of *D. melanogaster* and accepted hits with e-value < 0.001 as significantly homologous. We then used these *D. melanogaster* homologs as proxy in WebGestalt to assess enrichment of their associated *G. m. morsitans* homologs. We used the FDR corrected p-value ranked *D. melanogaster* homolog gene-sets of differentially expressed *G. m. morsitans* transcripts as input for the analysis [55]. We considered selection of 5–2000 Entrez Gene IDs, FDR < 0.05, 1000 permutations and 20 categories with the outputted leading-edge genes default parameters for the analysis. Through GSEA, we separated and identified significantly enriched non-redundant biological processes, cellular components and molecular function Gene Ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes, KEGG, PANTHER, Reactome, pathways and Database of Protein, Chemical and Genetic Interactions (BioGRID) network [56–61]. Next, we identified antennae or head (tissue) specific chemosensory genes by mapping the global most differentially (based on fold change)
and abundantly (based on CPM) or significantly expressed (based on p-value) transcripts in MA or volcano plots respectively using edgeR software package [52,62] in R software [63]. We considered chemosensory genes with fold changes (FC) $\geq 1.25$ as of chemosensory biological significance as previously documented [64].

Results

Expansions of chemosensory gene orthologs among tsetse fly species

We identified 60 each of Ors, Irs or Grs, 51 Obps, seven GluR and two Snmps (excluding isoforms) in D. melanogaster [43] and four CspS [40], with 58, 34, 13, 22, 2 and 3 orthologs (VectorBase gene trees, Release VB-2019-02) [44] respectively among the tsetse fly species (S1 Table). Cafe gene expansion analysis [45] revealed significant (P $< 0.05$, $\lambda = 2.60452e^{-7}$) accelerated expansions of several gene families/clusters including VBGT00190000010263 (Or71a and Or46a), VBGT00190000009736 (Ir75a,d, Ir31a, Ir84a and Ir64a) and VBGT00190000009994 (Obp83a-b) in G. m. morsitans, VBGT00840000047907 (Or67a,c, Or49a, Or92a, Or85b-c,f) and VBGT00190000013627 (Obp73a) in G. pallidipes, VBGT00190000012412 (Ir21a) and VBGT00190000010879 (Gr21a and Gr63a) carbon dioxide receptors orthologs [65] in G. f. fuscipes, VBGT00820000046003 (clumsy, Ir25a and Ir8a) in G. p. gambiensis and VBGT00190000013104 (Ir68a) in G. brevipalpis (S1 Table). No gene families were significantly expanded in G. austeni. We also identified several orthologs that were missing/absent in specific tsetse fly species (S1 Table). The Ir76b ortholog was absent in four tsetse fly species (G. p. gambiensis, G. m. morsitans, G. p. pallidipes and G. b. brevipalpis) while Gr33a was missing in G. b. brevipalpis. Both Gr32a and Gr68a were missing in G. b. brevipalpis and G. m. morsitans. The Gr64a-f, Gr5a, Gr43a, Obp56a/d/e and Or71a orthologs were absent in all tsetse fly species. The Snmp1, Or67d and Obp19a and Orco ortholog appeared to be conserved across all tsetse fly species. Our PCA analysis revealed a general positive correlation between tsetse species across four chemosensory groups (Ors, Irs, Grs or Obps). Additionally, Gr and Ir orthologs appeared to be positively correlated (S1 Fig panels B2 and B3) in relation to a unique G. m. morsitans cluster (S1 Fig panels A2 and A3).

Expression profiles of chemosensory ortholog transcripts in male G. m. morsitans antennae

The RNA-Seq of the antennae and head libraries yielded 23.3 to 17.9 million reads from respective libraries. We successfully mapped 51.0 to 69.6% of these reads onto G. m. morsitans transcripts where we established about 88.4% unique mappings of the reads to specific transcripts (Fig 1). We have summarized expressions profiles of the chemosensory orthologs in Fig 2. Orco, Or56a, 65a-c, Or47b and Or67b, and three G. m. morsitans specific orthologs (GMOY012254, GMOY009475, and GMOY006265) were among most abundantly expressed transcripts with Or33a-c orthologs exhibiting the least expression. Expressions of the members of the significantly expanded Ors gene families were marginal. Only six Gr orthologs were expressed among which Gr21a and Gr63a orthologs (carbon dioxide receptors) [65] and related two G. m. morsitans specific (GMOY013297 and GMOY013298) orthologs were abundantly expressed. The putative conserved core-receptors (Ir8a and Ir25a) and Ir41a were among the most abundantly expressed Irs orthologs. All but Ir75a-c expanded Ir orthologs were expressed. Most abundantly expressed Obp orthologs include Obp19a, lush, Obp28a, Obp83a-b Obp44a and two G. m. morsitans specific (GMOY012275 and GMOY013254) orthologs. Among these, Obp83a-b were among the significantly expanded Obp families. Both Snmps (Snmp 1 and Snmp 2) and Csp2 were also abundantly expressed.
Enriched pathways between male *G. m. morsitans* antennae and head libraries

Our Gene Set Enrichment Analysis (GSEA) of transcripts between the antennae and head libraries revealed several enriched pathways and processes between these tissues (Table 1, S2 Table). Our GoSlim GO analysis component of the GSEA assigned 85.4% of our transcripts to biological process, cellular components and molecular function ontologies (S2 Table). The most predominantly enriched biological processes between the antennae and head include metabolic processes, biological regulations, multicellular organismal processes, developmental processes and responses to stimuli. Most of these biological processes appeared to be localized in the membrane, macromolecular complex and nucleus cellular components, and were predominantly involved in protein binding, nucleic acid binding, ion binding and hydrolase activity molecular functions (S2 Table). More specifically, most enriched biological processes in the head were associated with vision, muscle activity and associated structural proteins and neuropeptide regulations, amino acid/nucleotide metabolism and circulatory system processes. The enriched cellular components were predominantly associated with vision and muscular functions. On the other hand, most enriched antennal biological processes were cilium-associated mechanoreceptors, chemo-sensation, neuronal controlled growth and
Fig 2. Expression profiles of *D. melanogaster* chemosensory gene orthologs in male *G. m. morsitans* antennae 72 hrs post feeding.

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### Table 1. Summary of Canonical Gene-set Enrichment Analysis (GSEA) of differentially expressed transcripts between male *G. m. morsitans* tsetse fly antennae and head transcripts.

| Functional Database | Tissue | Process ID | Annotation | General Function | Size | L | ES | NES | P Value | FDR |
|---------------------|--------|------------|------------|-----------------|------|---|----|-----|---------|-----|
| Gene                | Biological Head | GO:0005953 | Sensory perception of light stimulus | Vision | 59 | 22 | 0.898 | 1.883 | 0.000 | 0.000 |
| Gene                | Process | GO:0007186 | G-protein coupled receptor signaling pathway | Vision | 162 | 56 | 0.796 | 1.869 | 0.000 | 0.000 |
| GO:0032101 | Regulation of response to external stimulus | Vision | 101 | 8 | 0.801 | 1.833 | 0.000 | 0.000 |
| GO:0010927 | Cellular component assembly involved in morphogenesis | Muscle activity | 108 | 18 | 0.773 | 1.765 | 0.000 | 0.001 |
| GO:0042440 | Pigment metabolic process | Vision | 115 | 18 | 0.735 | 1.692 | 0.000 | 0.004 |
| GO:0006730 | Response to abiotic stimulus | Vision | 360 | 36 | 0.682 | 1.689 | 0.000 | 0.003 |
| GO:0003012 | Muscle system process | Muscle activity | 27 | 12 | 0.879 | 1.676 | 0.000 | 0.005 |
| GO:0044057 | Regulation of system process | Neuropeptide muscle regulations | 48 | 12 | 0.795 | 1.661 | 0.000 | 0.007 |
| GO:0004373 | Pigmentation | Vision | 103 | 18 | 0.706 | 1.610 | 0.003 | 0.025 |
| GO:0006730 | One-carbon metabolic process | Vision | 15 | 5 | 0.910 | 1.607 | 0.000 | 0.024 |
| GO:0003012 | Circulatory system process | Neuropeptide regulations | 40 | 12 | 0.784 | 1.590 | 0.005 | 0.032 |
| GO:0044782 | Cilium organization | Mechanoreception | 62 | 22 | -0.839 | 2.170 | 0.000 | 0.000 |
| GO:0031503 | Protein complex localization | Mechanoreception | 30 | 12 | -0.849 | 1.903 | 0.000 | 0.001 |
| GO:0007606 | Sensory perception of chemical stimulus | Chemo-reception | 124 | 57 | -0.628 | 1.806 | 0.000 | 0.006 |
| GO:0035218 | Leg disc development | Growth/differentiation | 87 | 15 | -0.665 | 1.781 | 0.000 | 0.007 |
| GO:0030705 | Cytoskeleton-dependent intracellular transport | Mechanoreception | 66 | 11 | -0.676 | 1.751 | 0.000 | 0.012 |
| GO:0030031 | Cell projection assembly | Mechanoreception | 112 | 35 | -0.624 | 1.742 | 0.005 | 0.011 |
| GO:0031099 | Regeneration | Repair/response to stress | 18 | 4 | -0.828 | 1.711 | 0.000 | 0.015 |
| Cellular | Head | GO:0019898 | Extrinsic component of membrane rhabdomere | Vision | 72 | 11 | 0.870 | 1.891 | 0.000 | 0.000 |
| Cellular | Component | GO:0016028 | rhabdomere | Vision | 34 | 17 | 0.955 | 1.886 | 0.000 | 0.000 |
| GO:0043292 | Contractile fiber | Muscle activity | 50 | 20 | 0.871 | 1.822 | 0.000 | 0.000 |
| GO:0015629 | Actin cytoskeleton | Vision/Muscle activity | 99 | 18 | 0.794 | 1.807 | 0.000 | 0.000 |
| GO:0098796 | Membrane protein complex | Vision | 233 | 10 | 0.690 | 1.689 | 0.000 | 0.001 |
| GO:0098878 | Actin-based cell projection | Vision | 22 | 4 | 0.861 | 1.600 | 0.002 | 0.012 |
| GO:0031984 | Organelle sub-compartment | Vision | 86 | 9 | 0.684 | 1.515 | 0.007 | 0.046 |
| Antennae | GO:0005929 | Cilium | Chemo-sensation/ Mechanoreception | 80 | 30 | -0.846 | 2.256 | 0.000 | 0.000 |
| GO:0031252 | Cell leading edge | Chemo-sensation | 52 | 28 | -0.811 | 2.005 | 0.000 | 0.000 |
| GO:0005815 | Microtubule organizing center | Mechanoreception/ Muscle activity | 111 | 20 | -0.666 | 1.849 | 0.000 | 0.001 |
| Molecular | Head | GO:0005516 | Calmodulin binding | Vision/Muscle activity | 43 | 6 | 0.834 | 1.706 | 0.000 | 0.009 |
| Molecular | Function | GO:0005549 | Odorant binding | Chemo-sensation | 49 | 35 | -0.843 | 2.170 | 0.000 | 0.000 |
| Pathway | KEGG | dme04745 | Phototransduction—fly—Drosophila melanogaster (fruit fly) | Vision | 25 | 14 | 0.954 | 1.772 | 0.000 | 0.000 |
| Analysis | Panther | P00057 | Wnt signaling pathway | Vision | 62 | 8 | 0.827 | 1.749 | 0.000 | 0.000 |
| P00031 | Inflammation mediated by chemokine and cytokine signaling pathway | Vision/Muscle activity | 26 | 5 | 0.895 | 1.716 | 0.000 | 0.002 |
| P00044 | Nicotinic acetylcholine receptor signaling pathway | Vision/Muscle activity | 38 | 9 | 0.845 | 1.705 | 0.000 | 0.002 |

(Continued)
differentiation, and regeneration/responses to stress, while enriched cellular components were associated with chemo-sensation, mechano-reception and muscular activities. Most enriched molecular functions in the head and antennae were associated with vision/muscular activities and chemo-sensation, respectively. The KEGG pathway analysis revealed enrichment of vision-associated pathways. Similarly, PANTHER pathway analysis also identified vision, in addition to neuropeptide signaling and muscular associated activities among the most enriched pathways in the head. We identified similar outcomes from our protein-protein interactions BIOGRID analysis in the head library. The Reactome pathway analysis identified vision and amino acids and derivative metabolism pathways predominating in the head transcriptome. We did not identify pathways or networks significantly enriched in the antennae library.

Table 1. (Continued)

| Functional Database | Tissue | Annotation | General Function | Size | L | ES | NES | P Value | FDR |
|---------------------|--------|------------|-----------------|------|---|----|-----|--------|-----|
| **Network Analysis** | **PPI_BIOGRID** | **Head** | **PPI_BIOGRID** | **M119** | **PPI_BIOGRID** | **M37** | **PPI_BIOGRID** | **M80** |
| R-DME-1852241 | Organelle biogenesis and maintenance | Vision | 38 | 4 | 0.904 | 1.778 | 0.000 | 0.000 |
| R-DME-2514856 | The phototransduction cascade | Vision | 12 | 6 | 0.961 | 1.687 | 0.000 | 0.025 |
| R-DME-5620920 | Cargo trafficking to the periciliary membrane | Vision | 15 | 4 | 0.956 | 1.683 | 0.000 | 0.018 |
| R-DME-5617833 | Cilium Assembly | Vision | 15 | 4 | 0.956 | 1.674 | 0.000 | 0.018 |
| R-DME-5620916 | VxPx cargo-targeting to cilium | Vision | 12 | 4 | 0.965 | 1.655 | 0.000 | 0.026 |
| R-DME-2514859 | Inactivation, recovery and regulation of the phototransduction cascade | Vision | 12 | 6 | 0.961 | 1.644 | 0.000 | 0.029 |
| R-DME-2187338 | Visual phototransduction | Vision | 14 | 6 | 0.957 | 1.644 | 0.000 | 0.025 |
| R-DME-76002 | Platelet activation, signaling and aggregation | Vision/Muscle activity | 47 | 9 | 0.784 | 1.640 | 0.000 | 0.024 |
| R-DME-71291 | Metabolism of amino acids and derivatives | Metabolism | 57 | 19 | 0.761 | 1.634 | 0.000 | 0.027 |
| R-DME-2672351 | Stimuli-sensing channels | Vision | 9 | 3 | 0.954 | 1.622 | 0.000 | 0.034 |
| R-DME-500792 | GPCR ligand binding | Vision | 14 | 4 | 0.920 | 1.618 | 0.002 | 0.034 |

*Non-Redundant*
Differentially expressed transcripts between male G. m. morsitans antennae and head libraries

Our search for both differentially (FC > 2) and abundantly expressed (CPM > 1) transcripts between the head and antennae libraries identified 2179 and 2158 transcripts respectively differentially expressed (FDR corrected p value < 0.05) between each library as summarized in our MA plot (Fig 3). Among these transcripts, at least 52 transcripts were most differentially and abundantly expressed (log FC > 2 and Average log CPM > 10) in both libraries. These transcripts were predominantly associated with vision, iron transport, metabolism and signal transduction in the head. In the antennae, the transcripts were involved in odor sensing and clearing, fatty acid synthesis and regulation of feeding behavior and locomotor activity (S3 Table). Analysis of both differentially (FC) and significantly expressed (p-value) transcripts between the head and antennae libraries identified 49 and 61 transcripts as most significantly expressed (FC >10 or < -5, and –log_{10} p-value > 25) in the head and antennae libraries respectively as summarized in our volcano plot (Fig 4). Overall, about 40 and 52 percent of the transcripts were associated with vision (head) and chemo-sensation (antennae) respectively. Most significantly expressed transcripts in the head library were functionally associated with energy mobilization, feeding, immunity, cytoskeleton integrity, amino acid metabolism, endocrine signaling and neuronal development and support. In the antennae, most significantly expressed transcripts were functionally associated chemo-sensation, metabolism, and cell proliferation, regulation of gene expression, signal transduction, anatomical integrity, neuron integrity/development and mechanoreception (S3 Table).

Differential expression of chemosensory gene transcripts between male G. m. morsitans antennae and head libraries

When we considered fold change greater than 1.25 as of biological chemosensory significance [64], most (> 90%) chemosensory transcripts showed significantly higher expressions in the antennae than in the head (Fig 5). Among these, significantly expressed chemosensory transcripts (p-value < 1e-20) in the antennae include several Obp (Lush, Obp19a, Obp28a, Obp59a, Obp83a/b and Obp84a), Ir (Ir25a, Ir31a, Ir40a, Ir41a, Ir64a, Ir75a, Ir76b, Ir84a, Ir8a and Ir92a), Or (Orco, Or7a, Or13a, Or43a, Or45a, Or47b, Or63a/c/d and Or85d), Gr (Gr21a), Csp [Csp2 (a10) and Csp4 (Phk-3)] and Snmp1 orthologs. Specifically, most significantly expressed transcripts were predominantly Obp orthologs. On the other hand, we identified a subset of obp (Obp8a, Clumsy, Obp99c Obp83cd), Or (Or85e, Or71a), Grs (Gr2a, Gr28b) and Csp4 (Phk-3) orthologs with significantly higher expression in the head than in the antennae libraries.

Discussion

In this study, we profiled expansions of chemosensory gene orthologs among six tsetse fly species/subspecies (G. pallidipes, G. m. morsitans, G. austeni, G. p. gambiensis, G. f. fuscipes and G. brevipalpis) and employed RNA-seq to discern differential expressions of the orthologs and associated proteins in antennae and head tissues male G. m. morsitans. Our café analysis for gene expansion revealed significant accelerated expansion of 4-methyl phenol and 4-propyl phenol responsive Or71a [66] in G. m. morsitans. The 4-methyl phenol and 4-propyl phenol are known G. m. morsitans and G. pallidipes attractants present in natural ox odor [17,67]. These findings probably account for the observed differential responses of these species to synthetic blends of these odors [68]. On the other hand, expansions of Ir75a,d, Ir31a, Ir84a and Ir64a orthologs in G. m. morsitans suggest differential odor-tuning and responses to acetic
Expanded chemosensory gene orthologs among selected tsetse species
Fig 3. MA plot showing abundantly and differentially expressed transcripts between the male *G. m. morsitans* head and antennae transcriptomes. Dots indicate points-of-interest that display individual transcript abundance (x axis) and fold-change (y axis). Red dots indicate transcripts with fold-changes of two or more (log₂ ≥ 1) and False Discovery Rate (FDR) corrected p values of less than 0.05 (significant) between the head and antennae transcriptomes. Black dots indicate transcripts with non-significant changes between the transcriptomes.

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Acetic acid component of the vertebrate breath is an attractant of most hematophagous vectors while 2-oxopentanoic acid elicits a landing response from *Anopheles gambiae* [74]. Whether there is enhanced attraction and landing behavior in *G. m. morsitans* in the presence of these kairomones remains to be determined. Expansion of Ir84a in *G. m. morsitans* may also indicate enhanced response to phenylacetaldehyde and male-male courtship [75] in this tsetse fly specie relative to the other species. Expansion of hunger responsive Obp83a ortholog [76] in *G. m. morsitans* suggest enhanced host seeking persistence in this specie relative to the other species. The *G. pallidipes* appears to be characterized by potentially muted responses to feeding stimulating hydroxycinnamic acids linked to missing Or71a [77], but enhanced responses to butanol, 2-heptanone and ketones lactones and phenolic compounds associated with the expanded Or49a [78,79], Or67a [80], Or85f [81] and Or85c [82] orthologs. The responses to butanol, lactones, ketones and phenolic compounds have been evaluated in development of baits used routinely in field control of *G. pallidipes*. Carbon dioxide receptors Gr21a and Gr63a orthologs [65] were expanded in *G. f. fusipes* and most abundantly expressed in male *G. m. morsitans* antennae. These findings are indicative of the heavier investment by *G. f. fusipes* than other tsetse flies in carbon dioxide detection and consequently host location [83]. The potential impact of the expansion (in *G. f. fusipes*) of the Ir21a receptor for cool avoidance behavior [84] is not clear, but may be tied to the humid and warm habitat preference in the *G. f. fusipes* lacustrine habitats. The Gr64a-f, Gr5a and Gr43a sugar receptor orthologs [85,86] were conspicuously absent in tsetse flies, consistent with our previous finding [40], a phenomenon attributable to exclusive sugar deficient blood diet in tsetse flies. The *G. brevipalpis* specific expansions of hygrosensory behavior mediating Ir68a ortholog [87] suggest potential behavioral responses to these and related odor cues specific to this tsetse fly. We did not identify expansion of Or67d in tsetse flies, contrary to previous reports [39,40].

We identified several missing/absent or conserved tsetse fly species specific orthologs with potential implications on respective tsetse species phenotypes. Absent Gr33a ortholog responsive to nonvolatile repellent chemicals, including N,N-diethyl-meta-toluamide (DEET) [88,89] in *G. brevipalpis* and marginal expression of Gr66a ortholog in male *G. m. morsitans* antennae, suggest diminished responses in these species to some repellents. This phenomenon is further supported by absence of another caffeine and DEET responsive Gr32a ortholog [88,89] and courtship pheromone associated Gr68a ortholog [90] in *G. brevipalpis* and *G. m. morsitans*. The missing Ir76b ortholog in four tsetse fly species (*G. p. gambiensis*, *G. m. morsitans*, *G. pallidipes* and *G. brevipalpis*) suggests that these tsetse species may have reduced responses to Ir76b ortholog mediated feeding preferences for amino acids [73] relative to remaining tsetse fly species. The conspicuous absence of Obp56a,d,e orthologs in tsetse flies, point to possible reduction in their responses to the associated pheromones [91]. Geosmin responsive Or56a ortholog [92] was most abundantly expressed Or after Orco in the *G. m. morsitans* antennae. Since Geosmin is a microbial odorant that alerts flies of presence of harmful microbes and induces avoidance behavior [92], the findings suggest potential repellence of tsetse flies by Geosmin and associated compounds, which can form a basis for a search for tsetse fly specific repellents. Conserved Gr2a, Gr28b and Gr66a orthologs across most species supports a notion of general aversion of salts [93], caffeine, DEET and some amino acids (theophylline, threonine and valine) [88,94–97] among the vectors. The Snmp1 ortholog
associated with detection of pheromones appears to be conserved across all the tsetse fly species, which in concert with similarly conserved Or67d and Orco orthologs, are functionally associated with detection of lipid-derived pheromones [98,99]. Other conserved pheromone responsive orthologs, include male-specific pheromone 11-cis-vaccenyl acetate (cVA)

Fig 4. Volcano plot showing abundantly and significantly expressed transcripts between the male *G. m. morsitans* head and antennae transcriptomes. Dots indicate points-of-interest that display fold-changes (x axis) and statistical significance (−log10 of p value, y axis) in transcripts between the head and antennae transcriptomes. Red dots indicate transcripts with fold-changes of two or more (log2 ≥ 1) and False Detection Rate (FDR) corrected p values of less than 0.05 and are indicate transcripts with significant changes between the transcriptomes. Black dots represent transcripts with non-significant changes between the transcriptomes.

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responsive lush and Obp19a [100] (absent in G. austeni) and l-carvone, 2-heptanone and acetophenone responsive Obp83a [101]. Lush, Or67d, Or83c and Obp83a were predominantly expressed in male G. m. morsitans antennae. We identified Ir93a ortholog in G. austeni contrary to previous findings [40]. Overall, we identified potential tsetse fly specific receptors and
semiochemicals/ligands for downstream functional validations that can be employed to expand the toolbox of tsetse fly attractants, repellents and regulators.

Our gene and pathway enrichment analyses suggest that male *G. m. morsitans* head and antennae are predominately involved with vision and olfaction (odor sensing and clearing) respectively. In addition to the classical and canonical olfaction pathways, we also established fatty acid synthesis and associated xenobiotic responsive cytochrome P450 (Cyp6g1/2, Cyp304a1) and Glutathione S transferase pathways preferentially enriched in the antennae. Similar observations have been made in cutworm moth (*Agrotis ipsilon*) antennae [102] and may indicate significant investment in odor/pheromone clearing [103], probably as a strategy for faster desensitization of antennae responses in the absence or disengagement with relevant cues. Other enriched pathways and transcripts included lush, lush-like Obp19a, Obp28a and Obp83a/b, Obp84a, Or7a and Snmp1 that are associated with responses to pheromones [91,104]. The antennae transcriptome appears to be dominated with abundant, differentially expressed Ir75a-c, Ir31a, Ir84a, Ir41a, Ir92a and Gr21a orthologs, functionally associated with responses to various odor cues including acetic acid, 2-oxopentanoic acid [70–72], pyridine, 1,4-diaminobutane, cadaverine, spermidine, pyrrolidine [72], phenylacetyaldehyde [26], ammonia [20] and carbon dioxide [65]. Some of the cues, such as butanol, carbon dioxide and acetic acid are documented odor cues in the breath of the tsetse fly vertebrate hosts and are actively employed by tsetse fly in host location [10,15], suggesting that the rest might perform similar functions in nature.

The antennae were also enriched with transcripts associated with cilium mechanoreceptors/locomotor activity, indicating possible significant role of antennae in the detection of kinetic energy (energy of movement, e.g. touch, sound, vibration, changing pressure) or potential energy (e.g. gravity) and hence guiding physical orientation of the fly. Stress induced neuronal controlled growth and differentiation and regeneration pathways were also enriched in the antennae, suggesting important role of the antennae in modulating responses of the fly to fluctuations in oxygen levels, temperature and redox state [105]. In addition to vision gene, the head was enriched with muscle and associated structural proteins, and energy mobilization potentially associated with feeding, as well as neuropeptide regulations associated with modification of nervous and endocrine systems. Most differential and abundantly expressed head specific chemosensory transcripts were also functionally associated with feeding. These included Obp8a involved in food perception [106] and host location [107], and Gr28a/b and Gr2a linked to regulation of aversion to high-salt associated diet [93]. Phenotypic roles of other head-specific chemosensory transcripts, such as Csp2 (a10) and Csp4 (Phk-3), Clumsy, Obp99c, Obp83cd, Or85e, Or71a and Csp4 (Phk-3), remain to be elucidated. Other than vision, olfaction and associated molecular processes, other processes appear to dominate physiological and molecular functions in the head and antennae libraries, respectively, indicating other functional roles of these tissues. Since these tissues (antenna and head) where extracted in the morning, the transcriptional responses coincided with the peak activity of the tsetse flies and hence reflect chemosensory and visual processes associated with host finding behavior predominant in that duration. Since our gene analyses were focused on antennae from male *G. m. morsitans*, our gene expression results were potentially biased toward male tsetse flies and *G. m. morsitans* subspecies. It would therefore be prudent to further assess for similar response in the remaining five tsetse fly species/subspecies, both gender and at different physiological states that influence their olfactory responses.

Conclusions

We identified tsetse fly specific chemosensory gene orthologs and their putative ligands, as potential candidates for downstream functional genomic and field validations. The validations
could yield new tsetse fly attractants, repellents and pheromones with potential in incremental improvements of current tsetse fly control strategies. We also identified major sensory pathways and processes potentially active in the tsetse fly antennae and head that can be exploited in modulating tsetse fly behavior.

**Supporting information**

S1 Fig. Principal Component Analysis (PCA)-based clustering of gene orthologs showing differences in number of expanded/contracted orthologs between the six tsetse fly species. (A) Clustering of chemosensory orthologs between tsetse species (B) Clustering of individual orthologs within chemosensory gene families.

(TIF)

S1 Table. Counts of chemosensory gene orthologs among fruit fly (*D. melanogaster*) and selected tsetse fly species.

(XLSX)

S2 Table. Canonical Gene-set Enrichment Analysis (GSEA) Gene Ontology, Kyoto Encyclopedia of Genes and Genomes (KEGG), Panther and Reactome pathways, and Protein-Protein Interactions BIOGRID network statistics for the differentially expressed transcripts between male *G. m. morsitans* antennae and head transcriptomes.

(XLSX)

S3 Table. Annotations of most abundantly or significantly differentially expressed transcripts between male *G. m. morsitans* antennae and head transcriptomes.

(XLSX)

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