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A mosquito-specific antennal protein is critical for the attraction to human odor in the malaria vector *Anopheles gambiae*

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ABSTRACT

Mosquitoes rely mainly on the sense of smell to decipher their environment and locate suitable food sources, hosts for blood feeding and oviposition sites. The molecular bases of olfaction involve multigenic families of olfactory proteins that have evolved to interact with a narrow set of odorants that are critical for survival. Understanding the complex interplay between diversified repertoires of olfactory proteins and ecologically-relevant odorant signals, which elicit important behaviors, is fundamental for the design of novel control strategies targeting the sense of smell of disease vector mosquitoes. Previously, large multigene families of odorant receptor and ionotropic receptor proteins, as well as a subset of odorant-binding proteins have been shown to mediate the selectivity and sensitivity of the mosquito olfactory system. In this study, we identify a mosquito-specific antennal protein (MSAP) gene as a novel molecular actor of odorant reception. MSAP is highly conserved across mosquito species and is transcribed at an extremely high level in female antennae. In order to understand its role in the mosquito olfactory system, we generated knockout mutant lines in *Anopheles gambiae*, and performed comparative analysis of behavioral and physiological responses to human-associated odorants. We found that MSAP promotes female mosquito attraction to human odor and enhances the sensitivity of the antennae to a variety of odorants. These findings suggest that MSAP is an important component of the mosquito olfactory system, which until now has gone completely unnoticed.

1. Introduction

Mosquito vectors impose an immense burden on human health, causing millions of infections and hundreds of thousands of deaths each year (Franklinos et al., 2019). At the frontline of current vector control strategies, insecticides have progressively lost their effectiveness due to the development of resistance and behavioral adaptations in mosquito populations (Coleman et al., 2017; Sanou et al., 2021). In this context, the development of alternative control strategies becomes a critical step toward future vector management programs. For this purpose, the olfactory system represents a relevant target to reduce the transmission of pathogens to humans, as it provides an interface between odorant cues from the environment and critical behaviors, such as host seeking (Carey and Carlson, 2011; McBride, 2016; Potter, 2014; Ray, 2015). The capacity to detect ecologically-relevant odor signals relies on a diversity of proteins that are expressed within specialized sensory structures, the olfactory sensilla, on two main types of peripheral olfactory organs, the antennae and the maxillary palps (Carey and Carlson, 2011; McBride, 2016; Potter, 2014; Ray, 2015).

The selectivity and sensitivity of the mosquito olfactory system depend on the expression of odorant receptors (ORs) (Carey et al., 2010; Wang et al., 2010) and ionotropic receptors (IRs) (Liu et al., 2010; Pitts et al., 2017) in distinct populations of olfactory sensory neurons (OSNs). Moreover, different classes of accessory proteins have been implicated in peripheral odorant reception mechanisms in the sensillum lymph, the aqueous medium surrounding the OSNs. Odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) have been suggested to ensure the liaison between the port of entry of odorants into the sensillum and the OSNs, contributing to binding, solubilizing and transporting odorants to the receptors across the sensillum lymph, with additional roles proposed for these soluble proteins (Pelosi et al., 2018; Rihani et al., 2021; Sun et al., 2018). Altogether, it is clear that the response profiles of different functional types of OSNs are mediated by the combinatorial expression of chemoreceptor- and binding proteins

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that evolved to interact with specific ranges of odorant ligands. However, despite an increasing knowledge of the function of chemoreceptors, disrupting the insect behavior in the field remains challenging (Wooding et al., 2020), possibly because the precise molecular mechanisms of odorant reception are not fully understood.

RNA sequencing studies of mosquito olfactory appendages have identified exhaustive repertoires of antennal and maxillary palps-enriched gene transcripts, providing an invaluable resource for identifying the whole set of genes mediating chemoreception processes in these important vector species (Hill et al., 2021; Leal et al., 2013; Matthews et al., 2016; Pitts et al., 2011). In transcriptome datasets, we identified a transcript that did not belong to any known olfactory gene family, ranked as the second most abundant in female antennae and which displayed a strong enrichment in this appendage in both *Anopheles gambiae* (Pitts et al., 2011) and *Aedes aegypti* (Matthews et al., 2016), raising the hypothesis that this gene might belong to an unidentified class of olfactory protein. At the sequence level, we found that this gene is highly conserved across mosquito species, and has no evolutionary-related orthologs in other organisms, including most insects, prompting us to name it a mosquito-specific antennal protein (MSAP) gene. Intrigued by these findings, we started investigating the function of MSAP in the mosquito olfactory system by generating targeted knockout (loss-of-function) mutant lines in *An. gambiae*, using CRISPR-Cas9 (Jinek et al., 2012). A comparative analysis (mutant versus wild-type) at the behavioral and physiological levels revealed that MSAP plays essential roles in female mosquito attraction to human odor and in the sensitivity of the antennae to individual human odor ligands. Overall, this exploratory study reveals MSAP as a "novel" class of olfactory protein, providing interesting new insight into the molecular mechanisms of olfaction in mosquitoes.

2. Methods

2.1. Insects

Laboratory colonies of *An. gambiae* (G3 strain), *Aedes aegypti* (Rockefeller strain) and *Culex quinquefasciatus* (Thai strain) were maintained under constant temperature (26 °C) and humidity (65% relative humidity), and at a 12 h:12 h light:dark cycle, as described previously (Omondi et al., 2019; Tallon et al., 2020; Taparia et al., 2017). For cloning and real-time quantitative PCR, 4–5 days post-eclosion (dpe) *An. gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus* females were used for tissue collection. For behavioral and electrophysiological experiments, 4–5 dpe *An. gambiae* females were tested in the first 3 h of the scotophase, during the peak of their host-seeking activity period (Das and Dimopoulos, 2008).

2.2. Genome identification of MSAP orthologs

Published RNA sequencing datasets of *An. gambiae* (Pitts et al., 2011) and *Ae. aegypti* (Matthews et al., 2016) were mined to identify transcript (s) that were: (1) highly abundant in female antennae; (2) enriched in female antennae versus non-olfactory tissues; and that (3) do not belong to any known olfactory gene family. The search revealed that the orthologous transcripts AGAP007976 (*An. gambiae*) and AAEL004301 (*Ae. aegypti*) satisfied all these criteria. The protein sequences were downloaded from VectorBase (https://vectorbase.org/vectorbase/app) and used as queries to perform homology Blast searches in the predicted protein databases at VectorBase and NCBI (https://www.ncbi.nlm.nih.gov/) using default settings. Putative MSAP orthologs were identified and combined to generate a multiple alignment file in Multalin (http://multalin.toulouse.inra.fr/multalin/). Manual reconstruction of full-length coding sequences from genome sequences was performed for a subset of MSAP orthologs, based on the consensus sequence derived from multiple alignment. The full-length MSAP transcript and protein sequences, as well as their main features are provided in SM-1 and SM-2, respectively.

2.3. Bioinformatics and phylogenetic analyses

MSAP amino-acid sequences were entered into SignalP 5.0 (https://services.healthtech.dtu.dk/service.php?SignalP-5.0) for predicting putative signal peptide cleavage sites, Jpred 4 (http://www.compbio.dundee.ac.uk/jpred/index.html) and Robetta (http://robetta.bakerlab.org/) for predicting putative protein secondary structures, and TMHMM server 2.0 (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) for predicting putative transmembrane domains. All analyses were performed using default settings. GeneDoc (https://nrbsc.org/gfx/genedoc) was used to calculate the amino-acid identity percentages between MSAP orthologs. A multiple alignment of mature proteins, without their signal peptides, was used to generate an entry file for phylogenetic analysis in MEGA 5.05 (https://www.megasoftware.net/). A consensus neighbor-joining tree was calculated using pairwise deletions under default settings. Branch support was assessed by bootstrap analysis based on 1000 replicates. The following MSAP orthologs were used in the analysis, with their respective VectorBase accession numbers indicated in brackets: (genus *Anopheles*) *An. gambiae* (AGAP007976), *An. albimanus* (AALB000316), *An. arabiensis* (AARA005150), *An. atroparvus* (AATE017637), *An. christyi* (ACHR003317), *An. coluzzii* (ACOM030722), *An. culicifacies* (ACUA018306), *An. darlingi* (ADAC010594), *An. dirus* (ADIR010994), *An. epiroticus* (AEPI004816), *An. farauti* (AFAF013789), *An. funestus* (AFUN006317), *An. maculatus* (AMAM012699), *An. melas* (AMEC018184), *An. merus* (AMEM017620), *An. minimus* (AMIN006292), *An. quadriannulatus* (AQUA000690), *An. stephensi* (ASTE006342), (genus *Aedes*) *Ae. aegypti* (AAEL004301), *Ae. albopictus* (AALF001366 and AALF015401) and (genus *Culex*) *Cx. quinquefasciatus* (CPIJ016648). The MSAP sequences used for phylogenetic analysis, including reconstructed sequences, are provided in SM-1. Note that additional MSAP sequences from different strains of the same species were not included in the phylogeny for clarity purposes, considering that these proteins were strictly identical to the same-species sequences already present in the tree. These sequences include: (*Anopheles* genus) *An. merus* AMEM21_010170.P19883 and AMEM21_010170.P19884, *An. coluzzii* ACMO_002983.P5663 and ACON007976, *An. arabiensis* AARA21_007987.P14934, *An. stephensi* ASTEI20_043343.P60626 and ASTEI01740, *An. albimanus* AALB20_029032, (*Aedes* genus) *Ae. albopictus* AALC636_010985.P15360, AALC636_014189.P19736, AALFPA_059616.P26249 and (*Culex* genus) *Cx. quinquefasciatus* CQUJHB001137.P1775.

2.4. Cloning of MSAP genes

RNA extraction of antennal tissues (from one hundred 4–5 dpe non-blood-fed females) was performed using Trizol (ThermoFisher, Waltham, USA), and the RNA samples were digested with TURBO DNase (ThermoFisher) and then immediately processed into complementary DNA (cDNA) using the iScript cDNA synthesis kit (BioRad, Hercules, USA). Gene-specific primers were designed to amplify the full-length cDNAs for *An. gambiae* (AGAP007976), *Ae. aegypti* (AAEL004301) and *Cx. quinquefasciatus* (CPIJ016648) *MSAP* genes. Primer sequences are provided in SM-3. PCR amplifications were carried out using Advantage HD DNA polymerase (Takara Bio, Kusatsu, Japan) in a final volume of 25 μl, following the instructions of the manufacturer. PCR products at the expected size were purified using QIAquick gel extraction kit (Qiagen, Hilden, Germany) and ligated into PCRII-TOPO plasmids (ThermoFisher). Ligation products were used to transform One Shot OmniMAX competent cells (ThermoFisher). Several clones were purified using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced (Eurofins Genomics, Ebersbeg, Germany).

2.5. Transcription profiles of MSAP genes by real-time quantitative PCR

One hundred non-blood-fed 4–5 dpe female mosquitoes (*An. gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus*) were used for tissue collection. Antennae, pooled maxillary palps and proboscis, legs and bodies were collected, and the RNA extracted and processed into cDNA as described in section 2.4. The cDNA samples were diluted ten times for real-time quantitative PCR experiments. Gene-specific primers (see SM-3) were designed using the OligoPerfect Primer Designer server (ThermoFisher) according to the following criteria: (1) an annealing temperature of 60 °C; (2) an amplicon size between 80 and 120 bps; and (3) an amplification product spanning an intron. The specificity of each pair of primers was confirmed by visualization of a single PCR amplicon at the expected size and by a single peak appearing in the melting curve analysis. Reactions were carried out using iQ SYBR Green Supermix (BioRad) in a final volume of 20 µl. Each reaction contained 10 µl of 2x iQ SYBR Green Supermix, 4 µl of diluted cDNA template, 2 µl of each primer (for a final primer concentration of 200 nM) and 4 µl ultrapure water. Master mixes were used to ensure better homogeneity, and individual reactions were distributed into Hard-Shell 96-well skirted low-profile PCR plates (BioRad). A standard cycling program of 95 °C for 2 min, 40 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 30 s, followed by a melt curve analysis in 0.5 °C increments from 55 °C to 95 °C for 20 s each, was run on a CFX96 real-time thermocycler (BioRad). Three technical replicates were performed on three independent biological samples, and control reactions with no DNA template were included for each set of primers. Analysis of real-time quantitative PCR data was performed using the CFX software (BioRad), and the relative transcript abundances were calculated using the comparative C_t method (ΔΔCt), as described by (Pelletier and Leal, 2011). The ribosomal protein S7 (RpS7) encoding gene was used as an endogenous control for normalization of the cDNA templates, and the pooled maxillary palps and proboscis sample was used as a calibrator.

2.6. Mutagenesis of Anopheles gambiae MSAP

The CRISPR-Cas9 system (Jinek et al., 2012) was used to generate targeted mutations in the coding sequence of the *An. gambiae MSAP* gene. After early unsuccessful attempts to obtain a mutation by direct co-injection of mosquito embryos with *in vitro*-synthesized guide RNA (gRNA) or a gRNA-coding plasmid with a Cas9-encoding plasmid, we opted for transgenic expression of both the gRNA and Cas9 components. To this end, we constructed pDSAR-U6-g, a transgenesis plasmid based on pDSAR (Volohonsky et al., 2015), in which an. *gambiae* U6 promoter (cloned from AGAP013557) drives the expression of a guide RNA template designed according to (Cong et al., 2013). A protospacer sequence (5′-GCGTACCGTTTCCAGCGAGA-3′), followed by a TGG protospacer-adjacent motif (PAM), was identified manually outside of naturally polymorphic regions of the coding sequence based on a multiple alignment of several independent clones (see section 2.4). The mutagenesis target site was selected in the first exon of *An. gambiae MSAP* in order to produce a truncated non-functional protein. We selected the protospacer sequence based on several criteria: (1) a conventional 20 nucleotides size; (2) the presence of a guanine at the 5′ end to allow transcription via a U6 promoter; (3) the presence of a PAM located immediately downstream the last base of the protospacer at the 3′ end; and (4) the presence of a native *Bst*XI recognition site at a position overlapping with the predicted CRISPR-mediated double-stranded DNA break, for genotyping purposes. A linker, obtained by annealing two oligonucleotides containing the protospacer sequence (5′-ccttGCGTACCGTTTCCA GCGAGA-3′ and 5′-aaacTCTCGCTGGAAACGGTACGC-3′) was ligated into the BbsI sites of pDSAR-U6-g to generate pDSAR-U6-MSAPgRNA. We injected embryos of the *An. gambiae* docking line X1 (a transgenic G3 strain) (Volohonsky et al., 2015) to generate a transgenic line expressing the gRNA. In parallel, we generated a transgenic line at the X1 locus expressing Cas9 under the control of the germline-specific vasa promoter, pDSAY-vasa-Cas9. The annotated pDSAR-U6-MSAPgRNA and pDSAY-vasa-Cas9 plasmid DNA sequences are provided in SM-4. The Cas9 and gRNA transgenic lines were crossed to combine the transgenes and induce mutagenesis in the germline of F_1 mosquitoes. F_1 males were backcrossed to wild-type females, separating the Cas9 and gRNA transgenes. F_2 female mosquitoes from the progeny of the backcross were again crossed to wild-type males, blood-fed, individualized and allowed to lay eggs. Individual F_2 females with a numerous progeny were screened by PCR for mutation in the *Bst*XI restriction site overlapping with the expected Cas9 cleavage site in *An. gambiae MSAP* (see genotyping in section 2.7). Out of nineteen screened females, fifteen had lost the *Bst*XI site and displayed a heterozygous genotype (see section 2.7). The mutagenesis target locus was amplified (GoTaq DNA polymerase, Promega, Madison, USA) (see primer sequences in SM-3), purified (QIAquick gel extraction kit, Qiagen) and sequenced (Eurofins Genomics), and two independent mutation events generating premature stop codons were selected to generate two mutant lines for behavioral experiments and electrophysiological recordings. A 4 bp deletion line was named $MSAP^1$ and a 5 bp insertion line was named $MSAP^3$.

2.7. Genotyping of Anopheles gambiae MSAP mutants

The progenies of F_2 mutant females with selected mutations (see mutagenesis in section 2.6) were used to establish the stable $MSAP^1$ and $MSAP^3$ mutant lines via four rounds of crossing and genotyping consisting of two backcrosses with wild-type insects followed by a selection of heterozygous and then homozygous mutants. Genotyping was carried out using the Phire Tissue Direct PCR Master Mix kit (ThermoFisher). Single legs (back leg for females and middle leg for males) were collected from individual virgin mosquitoes (2–3 days post-emergence), and placed into a 96-well plate for genomic DNA extraction and PCR amplification, following the instructions of the manufacturer. Gene-specific primers (see SM-3) were used to amplify a 339 bp *MSAP* genomic DNA fragment spanning the CRISPR-mediated double-stranded DNA break. After 32 cycles of amplification (98 °C for 5 min, 32 cycles at 98 °C for 10 s, 72 °C for 20 s and a final elongation at 72 °C for 1 min), the PCR products were digested with a FastDigest *Bst*XI (ThermoFisher) for 3 h at 37 °C. Digestion products were loaded onto a TAE-agarose gel for electrophoresis (85 V for 40 min), and the digestion profiles of individual insects were visualized using a DNA imaging system to reveal individual insect genotypes. After PCR and *Bst*XI digestion, the presence of an intact 339 bp amplicon indicated a mutant *MSAP* allele, whereas the presence of two distinct digestion products (200 bp and 139 bp) indicated a wild-type *MSAP* allele.

2.8. Behavioral assay

A comparative analysis of behavioral responses was carried out between mutant ($MSAP^1$ and $MSAP^3$), heterozygous ($MSAP^{1/+}$ and $MSAP^{3/+}$) and wild-type ($MSAP^+$) *An. gambiae* females (4–5 dpe) using a Y-tube olfactometer bioassay (see (Omondi et al., 2019) for details). The bioassay was employed to measure the attraction of female mosquitoes toward a previously developed synthetic human odor blend (Omondi et al., 2019) in a dose-dependent manner against a solvent control (pentane, >99%, Sigma-Adrich, St. Louis, USA). The blend was used in the absence of carbon dioxide. The blend and solvent were released using wick dispensers (4 cm × 1 cm; L:d; DAB Dental AB, Upplands Väsby, Sweden), which were placed inside 0.5 l glass wash bottles. Charcoal-filtered humidified air (at 1 l min^{-1}) was passed through the wash bottles to either of the two upwind arms of the olfactometer, via Teflon tubing. Prior to experiment, test insects were deprived of sugar for 24 h, and then transferred, in groups of five, to release cages for 4–6 h with continued *ad libitum* access to water. Then, the cages were moved into the downwind end of the olfactometer, and the mosquitoes allowed to acclimatize for 3 min, before they were released into the bioassay, where they were given a 5 min period to make a choice between the

arms containing the human odor or the solvent control. Only the insects entering either of the arms were considered for further analysis. The behavioral response to the human odor blend was calculated as a preference index, PI=(H–C)/(H + C), in which H is the total number of insects reaching the arm containing the synthetic human odor blend and C is the total number of insects reaching the control arm. Responses of host-seeking mosquitoes to the human odor blend were analyzed using generalized regression fitted with beta binomial distribution with AICc validation, followed by Tukey's honest significant difference test (JMP, Version 6, SAS Institute Inc., Cary, USA). The number of replicates used in the bioassay is given in SM-5.

2.9. Electroantennographic recordings

A full comparative analysis of antennal responses to individual odors was carried out between mutant ($MSAP^{3}$), heterozygous ($MSAP^{3/+}$) and wild-type ($MSAP^{+}$) *An. gambiae* females (4–5 dpe) using the electroantennogram (EAG) recording technique. Nine ecologically-relevant odorants, constituting a diverse range of chemical classes were used for the analysis: 3-octanol (CAS: 589-98-0; >95%), nonanal (CAS: 124-19-6; 95%), 6-methyl-5-heptene-2-one (sulcatone) (CAS: 110-93-0; 99%), linalool (CAS: 78-70-6; 97%), limonene (CAS: 138-86-3; 97%), nonanoic acid (CAS: 112-05-0; >97%), octanoic acid (CAS: 124-07-2; >99%), cadaverin (CAS: 462-94-2; >97%) and putrescin (CAS: 110-60-1; >99%). In addition, the mutant line $MSAP^{1}$ was tested against $MSAP^{+}$, but only using a single odorant, 3-octanol. Each odorant was prepared in decadic dilutions, from 10^{-1} to 10^{-5}, from neat compounds (from Sigma-Aldrich, St. Louis, USA) using hexane (Sigma-Aldrich, >95%) as the solvent. For odorant stimulation, a filter paper strip (2.5 cm × 1 cm) was inserted into a glass Pasteur pipette, and 10 μl of each dilution of each odorant was loaded onto the paper. The solvent was allowed to evaporate from the loaded pipettes under a fume hood for 30 min before use. Each stimulation pipette was used for a maximum of four individual recordings to avoid depletion of the odorant source. Among the test odorants, 3-octanol, nonanal, sulcatone, linalool and limonene are components of the synthetic human blend (see section 2.8).

For recordings, the whole head of an insect was used after the last segment of an antenna was cut off. A pulled glass microcapillary filled with Ringer's solution was inserted into the foramen of the mosquito head, constituting the reference electrode. A similar microcapillary was used to connect to the distal tip of the cut antenna, constituting the recording electrode. This electrode was attached to a pre-amplifier probe connected to a high impedance DC amplifier interface box (IDAC-2) (Ockenfels Syntech, Buchenbach, Germany). The prepared head was placed ca. 0.5 cm from a glass tube delivering a constant (1 l min^{-1}) charcoal-filtered humidified airflow. The odor stimulations consisted of a brief pulse of 0.5 s, in which the odorant was delivered into the main airflow via a CS-05 stimulus controller (Ockenfels Syntech). The antennal responses (in mV) were recorded and manually analyzed using the GC-EAD 2011 software (Ockenfels Syntech). To minimize recording biases across genotypes, mutant and heterozygous insects were always tested concomitantly with the wild-type control line, using the same odorant stimulation cartridges. In addition, the genotypes were rotated on each new set of stimulation cartridges of the same odorant, to avoid positional biases. Each recording included control stimulations by the solvent (hexane) at the beginning and at the end of every individual odor series. The individual antennal responses were normalized by subtracting the average control response from the neighboring hexane stimulations. The statistical analysis was performed using a Kruskal-Wallis test with P-values adjusted for multiple testing using Benjamini-Hochberg correction. The number of replicates used in the electrophysiological assay is given in SM-5.

2.10. Single sensillum recordings

Single sensillum recordings (SSRs) from capitate peg sensilla on the maxillary palp of wild type ($MSAP^{+}$) and homozygous mutant ($MSAP^{1}$ and $MSAP^{3}$) female mosquitoes were performed using a previously established protocol (Herre et al., 2022). Briefly, a single cold-anaesthetized female mosquito was placed ventrally on a microscope slide, covered with double-sided sticky tape. To secure the insect from moving, another piece of tape was put on the dorsal part of the body and pushed against the underlying tape. The maxillary palps were then gently mounted on the double-sided tape. Thereafter, the specimen was placed under a light microscope (700× magnification), which allowed visualization of the capitate peg sensilla for extracellular recordings.

Two electrolytically sharpened tungsten microelectrodes were prepared and mounted in holders (Ockenfels Syntech GmbH). The ground electrode was inserted into the eye of the mosquito, whereas the recording electrode, connected to a high-impedence universal single ended probe (Ockenfels Syntech GmbH), was inserted into a sensillum, using a piezo-controlled micro manipulator, until electrical contact with the sensillum was established. Signals were directed to an Intelligent Data Acquisition Controller (Ockenfels Syntech GmbH), and visualized on a computer screen. Quantification of spikes was carried out offline according to standard procedure (Ghaninia et al., 2019). The number of spikes counted during a 0.5 s stimulus delivery period was subtracted from that during a 0.5 s prestimulus period, and the outcome was multiplied by 2 to achieve the activity of each capitate peg sensilla -associated sensory neurons as a spikes/s measurement. Serial decadic dilutions of *R*-(−)-1-octen-3-ol (CAS: 3687-48-7, Penta Manufacturing, Livingston, USA) were diluted in paraffin oil, and a 15 μl aliquot was pipetted onto a filter paper (1 cm × 0.5 cm) placed inside a Pasteur pipette, and delivered as described above to assess the response of the B neuron within the capitate pegs (Ghaninia et al., 2019; Herre et al., 2022). Gas cylinders containing metered volumes of CO_2 (300, 600, 1200, 2400 or 4800 ppm) and oxygen (20%), balanced by nitrogen (Strandmöllen AB, Ljungby, Sweden) allowed for the dose-response analysis of the A neuron in the capitate pegs. The statistical analysis was performed using a generalized linear model followed by a Tukey HSD post-hoc test. The number of replicates used in the electrophysiological assay is given in SM-5.

3. Results and discussion

3.1. Identification of a putative novel class of mosquito olfactory protein

In an attempt to discover novel molecular components of the mosquito olfactory system, we explored published RNA sequencing datasets of *An. gambiae* (Pitts et al., 2011) and *Ae. aegypti* (Matthews et al., 2016) olfactory appendages to identify transcripts that are highly abundant and enriched in female antennae. That search led to a single conserved transcript, AGAP007976 in *An. gambiae* and AAEL004301 in *Ae. aegypti*, that ranked as the second most abundant in the antennae of both species, and had no sequence similarity to any known olfactory gene family. In order to verify that this gene was abundantly transcribed and enriched in the antennae of female mosquitoes, we used real-time quantitative PCR to compare the relative transcript levels in olfactory appendages (antennae and pooled maxillary palps and proboscis) and in non-olfactory tissues (legs and bodies) of three mosquito species. The experiment confirmed that AGAP007976 and AAEL004301, but also the *Cx. quinquefasciatus* ortholog CPIJ016648, are highly transcribed and enriched in female antennae when compared to other tissues (Fig. 1). These extremely high levels of transcription observed in the antennae were only rivaled by those of a small subset of OBPs (Matthews et al., 2016; Pitts et al., 2011), indicating a potential role of these genes in olfaction. Meanwhile, the relatively higher levels of transcription observed in maxillary palps relative to non-olfactory tissues could

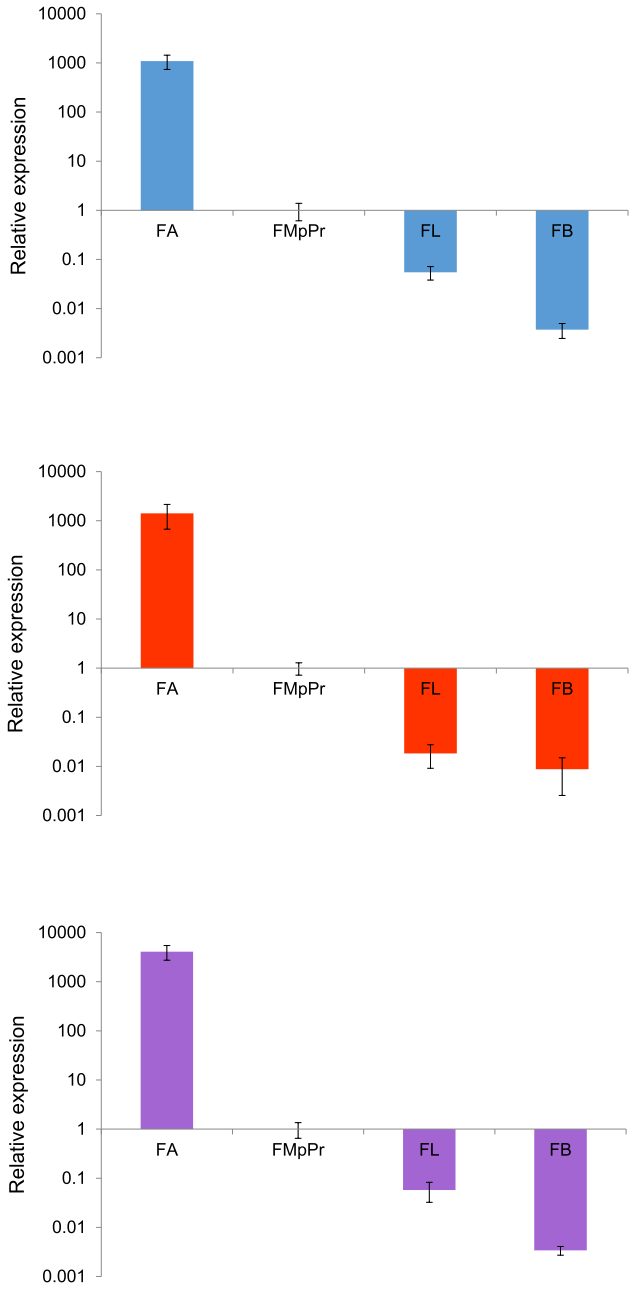

Fig. 1. Comparison of *MSAP* transcript levels in female antennae (FA), pooled maxillary palps and proboscis (FMpPr), legs (FL) and bodies (FB) in *Anopheles gambiae* (in blue), *Aedes aegypti* (in red) and *Culex quinquefasciatus* (in purple) by real-time quantitative PCR. The normalization of cDNA templates was achieved by using *RpS7* as an endogenous control, and the pooled maxillary palps and proboscis sample was used as a calibrator. A logarithmic scale (base 10) is used for the y-axis. Bars represent the standard error of the mean.

suggest that MSAP is also potentially expressed in this secondary olfactory organ. However, it is important to note here that these relative expression values reflect the *MSAP* transcript levels, not protein levels.

To find homologous MSAP sequences in mosquitoes and other insects we next performed Blast searches in protein databases. A single orthologous sequence was identified for every mosquito species with a genome available (MSAP sequences are provided in SM-1), with the exception of *Ae. albopictus* which harbors two copies encoding identical proteins but differing in their nucleotide sequences. These two highly-related paralogs likely originate from a recent duplication event, which is not surprising for a species, which displays the most dramatic expansions of gene families, including olfactory genes, across any mosquito species studied to date (Chen et al., 2015). Among other vector insects, a related sequence was found in the sand fly *Phlebotomus papatasi* (PPAI000118), but it was only a short partial sequence. Our effort to reconstruct a complete sequence led to a truncated 118 amino-acid long protein that aligns well with selected portions of mosquito MSAPs and shares around 40% identity with them, but it is unclear whether a full-length functional MSAP is present in this sand fly species. In other insects, only a few dipteran species were found to have a related hit in Blast searches (*Chironomus riparius* CAG9812101; *Polypedilum vanderplanki* KAG5666346; *Bradysia coprophila* XP_037050650 and XP_037030742; *Bradysia odoriphaga* KAG4073094 and KAG4067068; *Hermetia illucens* XP_037927035; *Contarinia nasturtii* XP_031627241; and *Clinio marinus* CRK93276), but these proteins only displayed moderate identity, typically around 35%, when compared with mosquito sequences. A multiple alignment showed a high level of sequence conservation across mosquito orthologs, with *An. gambiae* (AGAP007976) sharing 68% and 62% amino-acid identity with *Ae. aegypti* (AAEL004301) and *Cx. quinquefasciatus* (CPIJ0166648), respectively, while the two Culicinae orthologs were found to share 73% identity. These findings led us to name this particular class of gene as the *mosquito-specific antennal protein* (MSAP) gene. A phylogenetic tree of MSAPs revealed that *Anopheles* (18 species) orthologs cluster together (sharing between 70% and 100% amino-acid identity), while Culicinae (*Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*) orthologs form a distinct clade, reflecting the known evolutionary relationships across mosquito lineages (Fig. 2). The presence of a unique MSAP gene across mosquito species contrasts with the large diversified repertoires of previously characterized olfactory gene families. Moreover, the high level of conservation across orthologs strongly suggests that MSAPs ensure conserved function(s) in the antennae of different mosquito lineages.

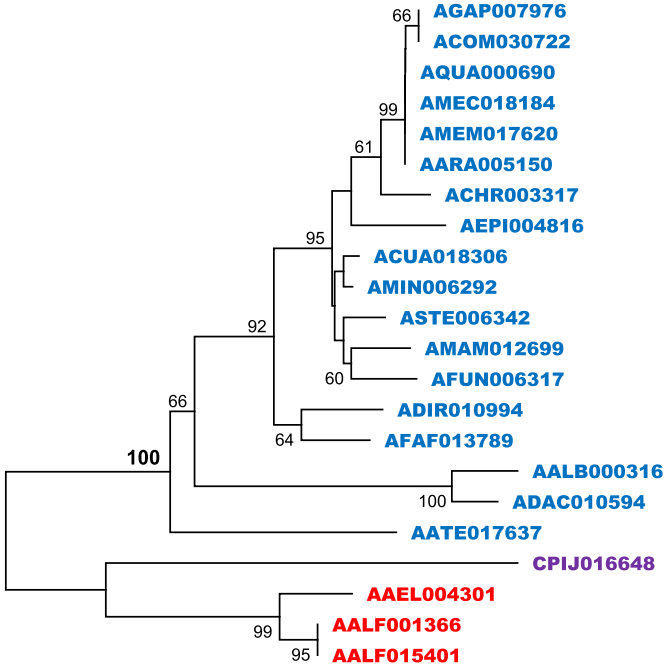

Fig. 2. Sequence comparison tree of MSAP orthologs. Twenty-two protein sequences were compiled for phylogenetic analysis. The tree was generated by the neighbor-joining method, with 1000 bootstrap replicates. *Anopheles* sequences are in blue, *Aedes* sequences are in red and the *Culex* sequence is in purple. The species name for each MSAP sequence is provided in the material and methods (section 2.3) and in SM-2. Only bootstrap values above 60% are indicated. The robust grouping of *Anopheles* MSAPs is supported by a 100% bootstrap value indicated in bold.

Since nothing was known about this class of protein, we examined the sequence features of MSAPs using bioinformatics servers to search for putative signal peptides, transmembrane domains and protein secondary structures. A signal peptide sequence was clearly predicted for each ortholog except in *An. dirus*, but a multiple alignment showed that a conserved cleavage motif (G/L) was present in all proteins (sequence features are provided in SM-2). The analysis also showed the absence of predicted transmembrane domains. The prediction of secondary structures revealed the presence of at least two putative alpha-helices comprising 13–17 residues, but the functional significance of these helices regarding the three dimensional structure of MSAPs is unknown. Finally, we identified a pattern of eight conserved cysteine residues in MSAPs, C_1-X_{26}-(C_2)-X_{26}-C_3-$X_{7\text{-}8}$-C_4-X_{12}-C_5-X_{23}-C_6-X_{50}-C_7-C_8, where the second cysteine (C_2) is present in all orthologs except in *An. epiroticus* and *Cx. quinquefasciatus*. A subset of these cysteine residues could be involved in the formation of disulfide bridges and contribute to the protein three-dimensional structure. We found no similarities between the cysteine patterns in MSAPs and those from other families of soluble olfactory proteins, such as the OBPs and CSPs (Pelletier and Leal, 2009, 2011). Taken together, these findings indicate that *MSAP* encodes a soluble protein possibly secreted into the sensillum lymph, the extracellular aqueous medium surrounding the OSNs dendrites where the reception of odorants occurs.

3.2. Functional characterization of MSAP in Anopheles gambiae

Sequence features and transcription profiles suggest that MSAPs might be involved in the mechanisms of odorant reception in the mosquito antennae. To understand the precise role of MSAP in the mosquito olfactory system, we used CRISPR-Cas9 (Jinek et al., 2012) to generate knockout lines in *An. gambiae*, via transgenic expression of both the guide RNA and Cas9 protein (Fig. 3). Genotyping of F_2 females revealed the presence of multiple individual mutation events, indicating that the mutagenesis strategy was successful. Sequencing of F_2 insects showed different deletion and insertion events at the expected double-stranded DNA break location. For illustration, a subset of deletion mutants, including $MSAP^1$ and $MSAP^2$, which harbor a 4 bp and a 5 bp deletion, respectively, and $MSAP^3$ and $MSAP^4$, which harbor a 5 bp insertion are displayed (Fig. 4). The $MSAP^1$ and $MSAP^3$ lines were selected for functional analyses, as both lines are expected to produce a non-functional truncated MSAP as a result of the frameshift induced by the mutation events. The selected mutant lines $MSAP^1$ and $MSAP^3$ were predicted to encode 56 and 59 amino-acid long mature proteins due to premature stop codons, to compare with the 170 residues that constitute the wild-type mature MSAP (after cleavage of the signal peptide sequence), further named $MSAP^+$. No morphological differences were observed between homozygous mutant and wild-type insects under a light microscope (700× magnification), including at the antennal and maxillary palp level.

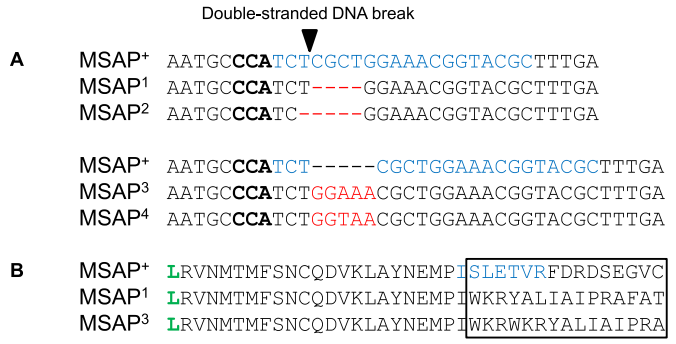

Fig. 4. Comparison of selected *Anopheles gambiae MSAP* mutant and wild-type sequences. The alignment of a portion of nucleotide (A) and protein (B) sequences of wild-type ($MSAP^+$) and mutant ($MSAP^1$, $MSAP^2$, $MSAP^3$ and $MSAP^4$) insects reveals the deletion and insertion events in four independent mutant lines. The protospacer is indicated in blue letters and the protospacer adjacent motif is in bold. Note that, for clarity, the protospacer sequence is presented in reverse orientation compared to Fig. 3. Red gaps and red letters indicate the nucleotides affected by the mutation events. The leucine residue in bold green is the first amino-acid of the mature protein located immediately after the signal peptide cleavage site. The box indicates the location of the frameshift induced by deletion and insertion events. The knockout lines $MSAP^1$ and $MSAP^3$ were selected for functional assays.

To investigate the potential role of MSAP in mosquito behavior, we focused on a well-characterized aspect of the mosquito biology, host-seeking. Previously, we have demonstrated that a synthetic human odor blend, comprising a subset of salient human-derived odorants, presented at ecological and physiological relevant concentrations, elicit strong behavioral responses (attraction) in host-seeking *An. coluzzii* females, when combined with carbon dioxide (Omondi et al., 2019). Here, we used the same Y-tube olfactometer strategy to compare the behavioral responses of wild-type ($MSAP^+$), heterozygous ($MSAP^{1/+}$ and $MSAP^{3/+}$) and homozygous mutant ($MSAP^1$ and $MSAP^3$) *An. gambiae*

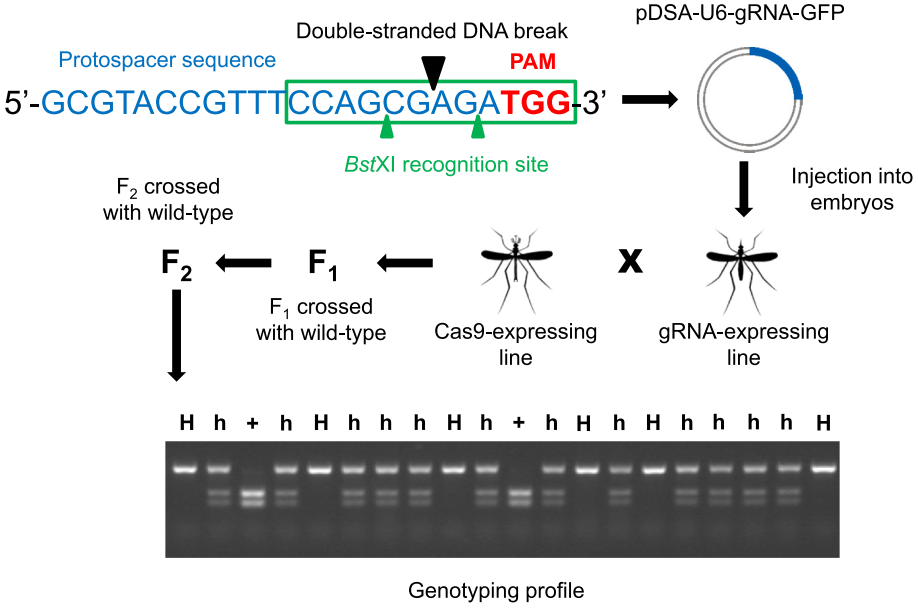

Fig. 3. Workflow of *Anopheles gambiae MSAP* mutagenesis. A protospacer sequence (in blue) followed by a protospacer-adjacent motif (in red), including a *Bst*XI recognition site (in green box) overlapping with the expected double-stranded DNA break region (black arrowhead), was selected for targeted mutagenesis. The protospacer sequence was cloned in-frame with a tracrRNA sequence into a pDSA vector for transgenesis, generating a guide RNA (gRNA) cassette under the control of a *U6* promoter. After co-injection of the DNA construct into mosquito embryos, gRNA-expressing mosquitoes were selected based on the expression of a fluorescent reporter and crossed with a Cas9-expressing line under the control of a *vasa* promoter. The F_1 and F_2 generations were then backcrossed with wild-type insects. The genotyping of individual mosquitoes was performed by PCR and *Bst*XI digestion, revealing specific band patterns for wild-type (+), as well as heterozygous (h) and homozygous (H) mutant mosquitoes. Note that only wild-type and heterozygous insects can be produced in the F_3 generation, but for illustration purposes, we have included all genotypes on the gel profile.

female mosquitoes to different concentrations of the same synthetic human blend, presented without carbon dioxide (Fig. 5) (see SM-5 for the number of replicates). The bioassay revealed a clear preference of MSAP^{+} control females to increasing doses of the odor blend, with a slight decline in attraction at the highest dose tested, likely reflecting habituation of the olfactory system (Glanzman, 2011) (Fig. 5). Both MSAP$^{1/+}$ and MSAP$^{3/+}$ heterozygous insects displayed a similar preference to the highest doses of the synthetic human blend as the MSAP^{+} control females (Fig. 5). In contrast, MSAP^{1} and MSAP^{3} homozygous mutant females were not attracted to the human blend at the higher doses, as compared to heterozygous and wild-type insects (Fig. 5). Rather, homozygous mutants consistently showed attraction to one of the lower dose tested compared to control and heterozygous mutant mosquitoes (Fig. 5). Overall, the comparative behavioral analysis revealed that the knockout of MSAP abolishes the attraction to high doses of the human odor in anthropophilic *An. gambiae* female mosquitoes (Fig. 5). This finding represents the first functional evidence that an olfactory protein other that a chemoreceptor contributes to odor-mediated host-seeking in mosquitoes.

The results of the behavioral assay indicate that MSAP plays an important role in the mosquito antennae, possibly by contributing to chemoreception processes in the sensillum lymph environment. To explore the functional contribution of MSAP in odorant reception, we used EAG recordings to compare the dose-dependent antennal responses of wild-type (MSAP^{+}), heterozygous (MSAP$^{3/+}$) and homozygous mutant (MSAP^{3}) female mosquitoes to individual odorants (Fig. 6) (see SM-5 for the number of replicates). This screening was complemented by testing MSAP^{1} against MSAP $^{+}$ as a control on a restricted panel of odorants (see SM-6). The panel was selected to represent diverse chemical classes, and included five odorants included in the synthetic human blend, which are all ligands for the OR pathway: 3-octanol (alcohol), nonanal (aldehyde), sulcatone (ketone), linalool (terpene alcohol) and limonene (terpene); and four odorants, which are ligands for the IR pathway: nonanoic acid and octanoic acid (carboxylic acids), as well as cadaverin and putrescin (amines). Importantly, we used a strategy in which mutants (and heterozygous insects) were tested concomitantly (during the same sessions) with wild-type controls on the same odorant set, to minimize recording biases across genotypes. The EAG analysis revealed a significant decrease of antennal responses to six odorants in MSAP^{3} insects, when compared with MSAP^{+} controls (Fig. 6). A similar trend was observed with MSAP^{1} when tested against MSAP^{+} with 3-octanol (SM-6), confirming that the targeted knockout is likely responsible for the observed difference. Interestingly, mutant insects displayed reduced responses to all nine odorants when stimulated with the highest doses (Fig. 6). In contrast, the antennal responses of MSAP$^{3/+}$ insects showed no significant difference when compared with MSAP^{+} controls (Fig. 6). Nevertheless, heterozygous insects also showed a slight reduction of response to most odorants when stimulated with the highest doses (Fig. 6). This slight decrease in heterozygous responses could indicate that the high levels of MSAP expression necessary for wild-type antennal responses might not be fully reached in heterozygous insects. Overall, the comparative EAG screening revealed that the knockout of MSAP affects the mechanisms of odorant reception, and suggests that MSAP plays a role in enhancing the sensitivity of the mosquito antennae in a context of high odorant concentrations. Interestingly, the reduction of antennal sensitivity was observed for all different types of odorants, suggesting that MSAP is widely distributed, and functions within both OR- and IR-expressing sensilla. This finding represents the first functional evidence that an olfactory protein contributes to both chemoreception pathways in mosquitoes.

To further explore the role of MSAP in odorant detection, we used SSRs to compare the dose-dependent maxillary palp responses of wild-type (MSAP^{+}) and homozygous mutant (MSAP^{1} and MSAP^{3}) female mosquitoes to carbon dioxide and (*R*)-1-octen-3-ol (Fig. 7). The SSR analysis revealed that MSAP is likely not involved in the reception of odorants in the maxillary palps, since no significant differences were found between the response profiles of the different genotypes to either ligand (CO_2, GLM: df = 2, F = 2,65, p = 0,074; (*R*)-1-octen-3-ol, df = 2, F = 0,79, p = 0,46).

3.3. The mode of action of MSAP is unknown

Behavioral and physiological experiments indicate that MSAP plays important role(s) in the mosquito olfactory system. From a functional perspective, the lack of attraction to the highest doses of human odor in mutant mosquitoes in the bioassay (Fig. 5) appears consistent with their reduced antennal sensitivity to the highest concentrations of individual odors in EAG recordings (Fig. 6 and SM-6). Indeed, the extreme sensitivity of the mosquito olfactory system to a narrow set of behavior-modifying odorants is thought to be critical for long-range orientation toward an odor source. Therefore, MSAP might be essential for the mosquito to perform different types of odorant-mediated behaviors. Interestingly, the most dramatic effects were observed when mutant insects were challenged with the highest doses of odors in both the olfactometer and electrophysiological assays. In this context, it seems reasonable to speculate that MSAP could be involved in some sort of gain

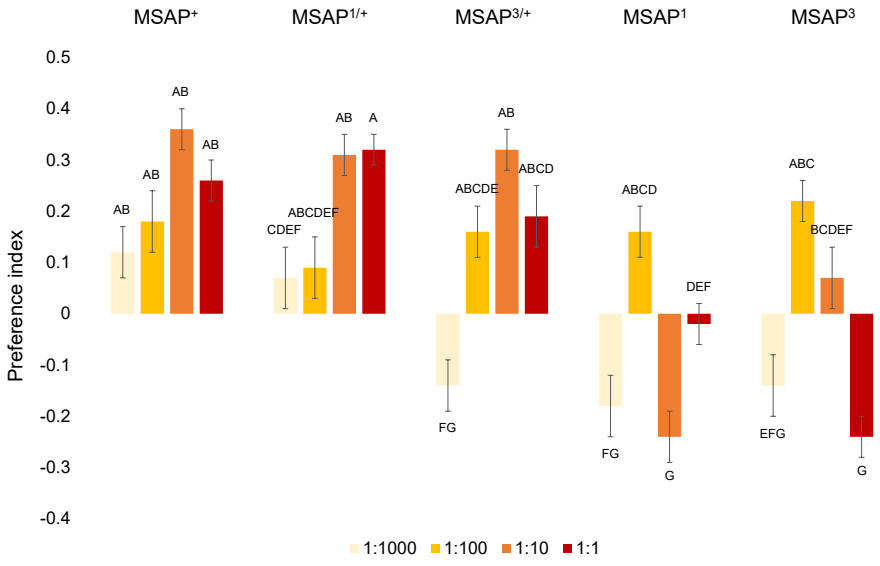

Fig. 5. Comparative analysis of behavioral responses of wild-type (MSAP^{+}), heterozygous (MSAP$^{1/+}$ and MSAP$^{3/+}$) and homozygous mutant (MSAP^{1} and MSAP^{3}), *Anopheles gambiae* females to four different concentrations of a synthetic human odor blend. The blend was presented *versus* a solvent control in a Y-tube dual-choice olfactometer. A preference index was calculated as the number of mosquitoes entering the human odor side minus those entering the control side, divided by the total number of mosquitoes entering either side. Bars represent the standard error of the mean. Letters indicate the level of significance for the response of each genotype to each dose, based on a Tukey's honest significant difference test. The number of replicates per genotype is provided in SM-5.

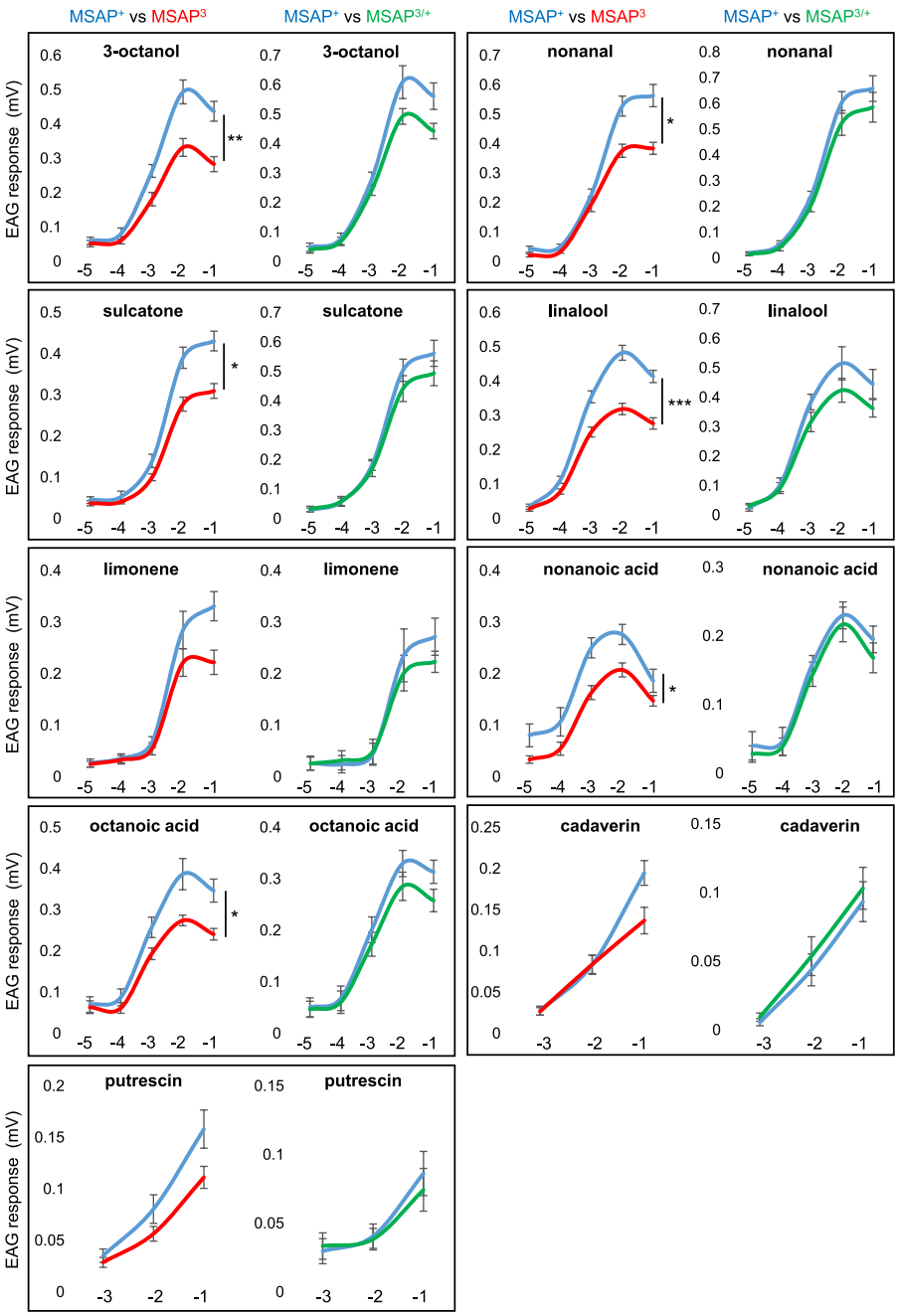

Fig. 6. Comparative analysis of antennal responses of wild-type ($MSAP^{+}$), heterozygous ($MSAP^{3/+}$) and homozygous mutant ($MSAP^{3}$), *Anopheles gambiae* females to nine individual odorants by electroantennographic recordings. Bars represent the standard error of the mean. Asterisks indicate significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) in the antennal responses between genotypes, based on a Kruskal-Wallis test. The number of replicates per genotype is provided in SM-5.

control function, by managing high levels of odorants in the mosquito antennae. However, at this stage, we can only speculate about the precise molecular mechanism(s) involved in that process. Considering the short size of MSAP (170 residues for the full-length mature protein), the presence of a binding pocket that could accommodate ligands from diverse chemical classes, such as those tested in this study appears unlikely. Similarly, the presence of interaction domains with other molecular actors of the OR and/or IR pathways, such as the OR co-receptor and IR co-receptors, also appears unlikely. Alternatively, we cannot exclude that MSAP is secreted into some intracellular compartment(s) of the sensilla with no direct access to the sensillum lymph environment, as previously observed with an abundant antennal *Drosophila melanogaster* OBP, DmelOBP19d. Contrarily to most other OBPs, this protein was found to localize within the outer space of coeloconic sensilla with no access to OSNs dendrites, but its potential role in olfaction is not known (Larter et al., 2016). In this scenario, MSAP might function in intracellular transduction mechanisms or in the maintenance of the sensilla structure and/or environment. To distinguish between the extracellular and intracellular hypotheses, additional experiments such as fluorescent *in situ* hybridization are needed to investigate the precise localization of MSAP in the mosquito olfactory system.

The ability to cope with high and rapidly fluctuating concentrations of odorants is fundamental for the temporal dynamic of the insect olfactory system. In the current picture of peripheral odorant reception, odorant signal termination (also called odorant signal inactivation or deactivation) is critical for preventing the continuous stimulation of OSNs, and for resetting the olfactory system in preparation for the detection of upcoming stimuli (Leal, 2013; Schmidt and Benton, 2020;

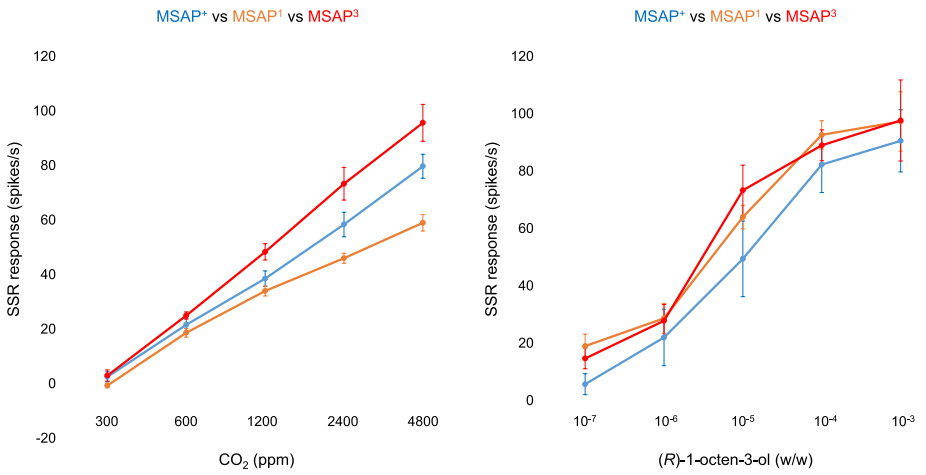

Fig. 7. Comparative analysis of maxillary palp responses of wild-type ($MSAP^{+}$) and homozygous mutant ($MSAP^{1}$ and $MSAP^{3}$) female *Anopheles gambiae* to carbon dioxide (CO_2) and (*R*)-1-octen-3-ol by single sensillum recordings. Bars represent the standard error of the mean. A generalized linear model followed by Tukey HSD post-hoc analysis revealed no significant differences between genotypes in their neuronal responses to CO_2 and (*R*)-1-octen-3-ol. The number of replicates per genotype is given in SM-5.

Suh et al., 2014). Previous experimental evidence supports the involvement of odorant-degrading enzymes (ODEs) (Chertemps et al., 2015; Steiner et al., 2017) and also some OBPs (Scheuermann and Smith, 2019) in odorant signal termination in *Drosophila melanogaster*. Nevertheless, it is still not known how ODEs and some OBPs (and possibly some other unknown molecular actors) precisely interact to ensure proper odorant clearance in the sensillum lymph of different sensilla types (Leal, 2013; Schmidt and Benton, 2020; Suh et al., 2014). In mosquitoes, the high levels of expression of a number of candidate ODEs and OBPs (thirteen *OBPs* and three putative *ODEs* appear in the top 20 of the most transcribed genes in *An. gambiae* female antennae (Pitts et al., 2011)) suggest that they could be involved in clearing high doses of odors in the OSNs environment. Previous studies have investigated the function of highly abundant antennal OBPs in odorant reception, illustrating different potential roles for these proteins. In *Drosophila melanogaster*, the knockout of *OBP28a* showed an increase in the OSNs responses (in single-sensillum recording experiments) to a number of odorants in mutants when compared with controls, in particular during the initial phase of OSN activation (Larter et al., 2016). The authors suggested that this protein could function as a buffer against rapid increases in odor levels, by binding some of the ligands before the activation of the chemoreceptor, thus providing a molecular mechanism for gain control. In contrast, the knockdown of two major mosquito antennal *OBP* transcripts (encoding orthologous AgamOBP1 and CquiOBP1) showed weaker antennal responses in dsRNA-injected insects (in EAG) when compared with controls, providing evidence for the role of this protein in promoting antennal sensitivity to specific odors (Biessmann et al., 2010; Pelletier et al., 2010). The MSAP knockout physiological phenotype resembles the OBP1 knockdown phenotype but seems to affect the sensitivity of antennal responses only at the higher doses of stimuli and, contrarily to OBP1, displays no selectivity toward odor ligands. Although the mode of action is still unknown, our findings suggest that MSAP could mediate a distinct molecular mechanism of gain control in mosquitoes, possibly concomitant with regular signal inactivation processes ensured by putative ODEs and possibly some OBPs. To further investigate the precise role(s) of MSAP in odorant reception, it would be interesting to fully characterize the biochemical properties of this unique type of protein in relation to potential olfactory functions.

4. Conclusion

The rapid evolution of insect olfactory gene families has resulted in diverse species-specific repertoires that reflect the necessity of insects to decipher distinct chemical environments and adapt to different ecological niches. In contrast, MSAP is found exclusively in mosquitoes (although a distant homolog is present in a few other dipteran species) and represents an unexpected case of a lineage-specific type of insect olfactory protein. From an evolutionary perspective, the absence of orthologs in other insects raises interesting questions about the past trajectory of this gene, but it is currently unclear how and why it is absent in all other insect lineages, as well as in most other dipteran insects. From a functional perspective, MSAP displays unique characteristics for an insect soluble olfactory protein: it enhances the sensitivity of the antennae to a large diversity of odors, including ligands from both the OR and IR pathways, and directly regulates female attraction behavior toward human odor. The ability to influence the reception of a wide range of odorant ligands, coupled with the extremely high transcription levels observed in the antennae, suggests that MSAP could be expressed in all different types of olfactory sensilla and could contribute to most types of odorant-driven behaviors. The functional evidence at both physiological and behavioral levels indicates that MSAP could mediate a molecular mechanism of gain control, by managing high doses of odors in the antennae, but the precise mode of action is still unknown.

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Author's contributions

Julien Pelletier: Conceptualization, methodology, investigation, writing and editing; Mengistu Dawit: Investigation, formal analysis and editing; Majid Ghaninia: Investigation, formal analysis and editing; Eric Marois: Methodology, investigation and editing; Rickard Ignell: Conceptualization, methodology and editing.

Submission declaration and verification

The research manuscript is entirely original and has not been submitted for publication elsewhere. It has been approved by all authors. It will not be published elsewhere after publication.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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List of abbreviations

MSAP	mosquito-specific antennal protein
OSN	olfactory sensory neuron
OR	odorant receptor
IR	ionotropic receptor
OBP	odorant-binding protein
CSP	chemosensory protein
ODE	odorant-degrading enzyme
CRISPR	clustered regularly interspaced palindromic repeats
Cas9	CRISPR associated nuclease 9
PAM	protospacer adjacent motif
EAG	electroantennographic
SSR	single sensillum recording

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2023.103988.

References

Biessmann, H., Andronopoulou, E., Biessmann, M.R., Douris, V., Dimitratos, S.D., Eliopoulos, E., Guerin, P.M., Iatrou, K., Justice, R.W., Kröber, T., Marinotti, O., Tsitoura, P., Woods, D.F., Walter, M.F., 2010. The *Anopheles gambiae* odorant binding protein 1 (AgamOBP1) mediates indole recognition in the antennae of female mosquitoes. PLoS One 5 (3), e9471. https://doi.org/10.1371/journal.pone.0009471.

Carey, A.F., Carlson, J.R., 2011. Insect olfaction from model systems to disease control. Proc. Natl. Acad. Sci. U.S.A. 108, 12987–12995. https://doi.org/10.1073/pnas.1103472108.

Carey, A.F., Wang, G., Su, C.Y., Zwiebel, L.J., Carlson, J.R., 2010. Odorant reception in the malaria mosquito *Anopheles gambiae*. Nature 464, 66–71. https://doi.org/10.1038/nature08834.

Chen, X.G., Jiang, Xuanting, Gu, J., Xu, M., Wu, Y., Deng, Y., Zhang, C., Bonizzoni, M., Dermauw, W., Vontas, J., Armbruster, P., Huang, X., Yang, Y., Zhang, H., He, W., Peng, H., Liu, Y., Wu, K., Chen, J., Lirakisi, M., Topalis, P., Van Leeuwen, T., Hall, A. B., Jiang, Xiaofang, Thorpe, C., Mueller, R.L., Sun, C., Waterhouse, R.M., Yan, G., Tu, Z.J., Fang, X., James, A.A., 2015. Genome sequence of the Asian tiger mosquito, *Aedes albopictus*, reveals insights into its biology, genetics, and evolution. Proc. Natl. Acad. Sci. U.S.A. 112, E5907–E5915. https://doi.org/10.1073/pnas.1516410112.

Chertemps, T., Younus, F., Steiner, C., Durand, N., Coppin, C.W., Pandey, G., Oakeshott, J.G., Maïbèche, M., 2015. An antennal carboxylesterase from *Drosophila melanogaster*, esterase 6, is a candidate odorant-degrading enzyme toward food odorants. Front. Physiol. 6, 1–9. https://doi.org/10.3389/fphys.2015.00315.

Coleman, M., Hemingway, J., Gleave, K.A., Wiebe, A., Gething, P.W., Moyes, C.L., 2017. Developing global maps of insecticide resistance risk to improve vector control. Malar. J. 16, 1–9. https://doi.org/10.1186/s12936-017-1733-z.

Cong, L., Ran, A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P., Wu, X., Jiang, W., Marraffini, L., Zhang, F., 2013. Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819–823. https://doi.org/10.1126/science.1231143.

Das, S., Dimopoulos, G., 2008. Molecular analysis of photic inhibition of blood-feeding in *Anopheles gambiae*. BMC Physiol. 8, 1–19. https://doi.org/10.1186/1472-6793-8-23.

Franklinos, L.H.V., Jones, K.E., Redding, D.W., Abubakar, I., 2019. The effect of global change on mosquito-borne disease. Lancet Infect. Dis. 19, e302 https://doi.org/10.1016/S1473-3099(19)30161-6 e312.

Ghaninia, M., Majeed, S., Dekker, T., Hill, S.R., Ignell, R., 2019. Hold your breath - differential behavioral and sensory acuity of mosquitoes to acetone and carbon dioxide. PLoS One 14, 1–14. https://doi.org/10.1371/journal.pone.0226815.

Glanzman, D.L., 2011. Olfactory habituation: fresh insights from flies. Proc. Natl. Acad. Sci. U.S.A. 108, 14711–14712. https://doi.org/10.1073/pnas.1111230108.

Herre, M., Goldman, O.V., Lu, T.C., Caballero-Vidal, G., Qi, Y., Gilbert, Z.N., Gong, Z., Morita, T., Rahiel, S., Ghaninia, M., Ignell, R., Matthews, B.J., Li, H., Vosshall, L.B., Younger, M.A., 2022. Non-canonical odor coding in the mosquito. Cell 185, 3104–3123. https://doi.org/10.1016/j.cell.2022.07.024 e28.

Hill, S.R., Taparia, T., Ignell, R., 2021. Regulation of the antennal transcriptome of the dengue vector, *Aedes aegypti*, during the first gonotrophic cycle. BMC Genom. 22, 1–19. https://doi.org/10.1186/s12864-020-07336-w.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E., 2012. A programmable dual-RNA – guided. Science 337, 816–822. https://doi.org/10.1126/science.1225829.

Larter, N.K., Sun, J.S., Carlson, J.R., 2016. Organization and function of *Drosophila* odorant binding proteins. Elife 5, 1–22. https://doi.org/10.7554/eLife.20242.

Leal, W.S., 2013. Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. Annu. Rev. Entomol. 58, 373–391. https://doi.org/10.1146/annurev-ento-120811-153635.

Leal, W.S., Choo, Y.M., Xu, P., Da Silva, C.S.B., Ueira-Vieira, C., 2013. Differential expression of olfactory genes in the southern house mosquito and insights into unique odorant receptor gene isoforms. Proc. Natl. Acad. Sci. U.S.A. 110, 18704–18709. https://doi.org/10.1073/pnas.1316059110.

Liu, C., Pitts, R.J., Bohbot, J.D., Jones, P.L., Wang, G., Zwiebel, L.J., 2010. Distinct olfactory signaling mechanisms in the malaria vector mosquito *Anopheles gambiae*. PLoS Biol. 8, 27–28. https://doi.org/10.1371/journal.pbio.1000467.

Matthews, B.J., McBride, C.S., DeGennaro, M., Despo, O., Vosshall, L.B., 2016. The neurotranscriptome of the *Aedes aegypti* mosquito. BMC Genom. 17, 1–20. https://doi.org/10.1186/s12864-015-2239-0.

McBride, C.S., 2016. Genes and odors underlying the recent evolution of mosquito preference for humans. Curr. Biol. 26, R41–R46. https://doi.org/10.1016/j.cub.2015.11.032.

Omondi, A.B., Ghaninia, M., Dawit, M., Svensson, T., Ignell, R., 2019. Age-dependent regulation of host seeking in *Anopheles coluzzii*. Sci. Rep. 9, 1–9. https://doi.org/10.1038/s41598-019-46220-w.

Pelletier, J., Guidolin, A., Syed, Z., Cornel, A.J., Leal, W.S., 2010. Knockdown of a mosquito odorant-binding protein involved in the sensitive detection of oviposition attractants. J. Chem. Ecol. 36, 245–248. https://doi.org/10.1007/s10886-010-9762-x.

Pelletier, J., Leal, W.S., 2011. Characterization of olfactory genes in the antennae of the Southern house mosquito, *Culex quinquefasciatus*. J. Insect Physiol. 57, 915–929. https://doi.org/10.1016/j.jinsphys.2011.04.003.

Pelletier, J., Leal, W.S., 2009. Genome analysis and expression patterns of odorant-binding proteins from the Southern House mosquito *Culex pipiens quinquefasciatus*. PLoS One 4. https://doi.org/10.1371/journal.pone.0006237.

Pelosi, P., Iovinella, I., Zhu, J., Wang, G., Dani, F.R., 2018. Beyond chemoreception: diverse tasks of soluble olfactory proteins in insects. Biol. Rev. 93, 184–200. https://doi.org/10.1111/brv.12339.

Pitts, J., Derryberry, S.L., Zhang, Z., Zwiebel, L.J., 2017. Variant ionotropic receptors in the malaria vector mosquito *Anopheles gambiae* tuned to amines and carboxylic acids. Sci. Rep. 7, 1–11. https://doi.org/10.1038/srep40297.

Pitts, R.J., Rinker, D.C., Jones, P.L., Rokas, A., Zwiebel, L.J., 2011. Transcriptome profiling of chemosensory appendages in the malaria vector *Anopheles gambiae* reveals tissue- and sex-specific signatures of odor coding. BMC Genom. 12, 271. https://doi.org/10.1186/1471-2164-12-271.

Potter, C.J., 2014. Stop the biting: targeting a mosquito's sense of smell. Cell 156, 878–881. https://doi.org/10.1016/j.cell.2014.02.003.

Ray, A., 2015. Reception of odors and repellents in mosquitoes. Curr. Opin. Neurobiol. 34, 158–164. https://doi.org/10.1016/j.conb.2015.06.014.

Rihani, K., Ferveur, J.F., Briand, L., 2021. The 40-year mystery of insect odorant-binding proteins. Biomolecules 11. https://doi.org/10.3390/biom11040509.

Sanou, A., Nelli, L., Guelbéogo, W.M., Cissé, F., Tapsoba, M., Ouédraogo, P., Sagnon, N., Ranson, H., Matthiopoulos, J., Ferguson, H.M., 2021. Insecticide resistance and behavioural adaptation as a response to long-lasting insecticidal net deployment in malaria vectors in the Cascades region of Burkina Faso. Sci. Rep. 11, 1–14. https://doi.org/10.1038/s41598-021-96759-w.

Scheuermann, E., Smith, D., 2019. Odor-specific deactivation defects in a *Drosophila* odorant-binding protein mutant. Genetics 213, 897–909. https://doi.org/10.1534/genetics.119.302629.

Schmidt, H.R., Benton, R., 2020. Molecular mechanisms of olfactory detection in insects: beyond receptors: insect olfactory detection mechanisms. Open Biol 10. https://doi.org/10.1098/rsob.200252.

Steiner, C., Bozzolan, F., Montagné, N., Maïbèche, M., Chertemps, T., 2017. Neofunctionalization of "Juvenile Hormone Esterase Duplication" in *Drosophila* as an odorant- degrading enzyme towards food odorants. Sci. Rep. 7, 18–20. https://doi.org/10.1038/s41598-017-13015-w.

Suh, E., Bohbot, J.D., Zwiebel, L.J., 2014. Peripheral olfactory signaling in insects. Curr. Opin. Insect Sci. 6, 86–92. https://doi.org/10.1016/j.cois.2014.10.006.

Sun, J.S., Xiao, S., Carlson, J.R., 2018. The diverse small proteins called odorant-binding proteins. Open Biol 8. https://doi.org/10.1098/rsob.180208.

Tallon, A., Lorenzo, M., Moreira, L., Villegas, L.E.M., Hill, S., Ignell, R., 2020. Dengue infection modulates locomotion and host seeking in *Aedes aegypti*. PLoS Neglected Trop. Dis. 14, 1–20. https://doi.org/10.1371/journal.pntd.0008531.

Taparia, T., Ignell, R., Hill, S.R., 2017. Blood meal induced regulation of the chemosensory gene repertoire in the southern house mosquito. BMC Genom. 18, 1–9. https://doi.org/10.1186/s12864-017-3779-2.

Volohonsky, G., Terenzi, O., Soichot, J., Naujoks, D.A., Nolan, T., Windbichler, N., Kapps, D., Smidler, A.L., Vittu, A., Costa, G., Steinert, S., Levashina, E.A., Blandin, S.

A., Marois, E., 2015. Tools for *Anopheles gambiae* transgenesis. G3 Genes, Genomes, Genet. 5, 1151–1163. https://doi.org/10.1534/g3.115.016808.

Wang, G., Carey, A.F., Carlson, J.R., Zwiebel, L.J., 2010. Molecular basis of odor coding in the malaria vector mosquito *Anopheles gambiae*. Proc. Natl. Acad. Sci. U.S.A. 107, 4418–4423. https://doi.org/10.1073/pnas.0913392107.

Wooding, M., Naudé, Y., Rohwer, E., Bouwer, M., 2020. Controlling mosquitoes with semiochemicals: a review. Parasites Vectors 13, 1–20. https://doi.org/10.1186/s13071-020-3960-3.