

SHORT REPORT

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Female *Anopheles gambiae* antennae: increased transcript accumulation of the mosquito-specific odorant-binding-protein OBP2

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Abstract

Background: New interventions are required to optimally and sustainably control the *Anopheles* sp. mosquitoes that transmit malaria and filariasis. The mosquito olfactory system is important in host seeking (transmission) and mate finding (reproduction). Understanding olfactory function could lead to development of control strategies based on repelling parasite-carrying mosquitoes or attracting them into a fatal trap.

Findings: Our initial focus is on odorant binding proteins with differential transcript accumulation between female and male mosquitoes. We report that the odorant binding protein, OBP2 (AGAP003306), had increased expression in the antennae of female vs. male *Anopheles gambiae sensu stricto* (G3 strain). The increased expression in antennae of females of this gene by quantitative RT-PCR was 4.2 to 32.3 fold in three independent biological replicates and two technical replicate experiments using *A. gambiae* from two different laboratories. OBP2 is a member of the vast OBP superfamily of insect odorant binding proteins and belongs to the predominantly dipteran clade that includes the *Culex* oviposition kairomone-binding OBP1. Phylogenetic analysis indicates that its orthologs are present across culicid mosquitoes and are likely to play a conserved role in recognizing a molecule that might be critical for female behavior.

Conclusions: OBP2 has increased mRNA transcript accumulation in the antennae of female as compared to male *A. gambiae*. This molecule and related molecules may play an important role in female mosquito feeding and breeding behavior. This finding may be a step toward providing a foundation for understanding mosquito olfactory requirements and developing control strategies based on reducing mosquito feeding and breeding success.

Keywords: *Anopheles gambiae*, Odorant, Binding, Protein, Antennae

Findings

Research hypothesis

Factors that influence mosquito fitness, especially host seeking and mate finding are complex and modulated by multiple cues, of which olfactory cues are most important [1-4]. Detection of odor molecules requires odorant binding proteins (OBPs) that are abundant in antennal chemosensilla [5,6]. OBPs are low molecular weight soluble proteins that bind and transport odor molecules from sensillae to G-protein-coupled receptors in olfactory sensory neurons [6]. The finding of receptor

AgamOBP1 binding to its ligand indole demonstrated the significance of OBPs in odor recognition [7]. Understanding olfactory function could lead to development of malaria control strategies based on repelling *Plasmodium* sp. carrying *Anopheles* mosquitoes or attracting them into a fatal trap. A first step is assessment of expression of olfactory system associated genes [7-10]. There is sexually dimorphic expression of OBPs in *Anopheles* mosquitoes and *Drosophila melanogaster* [11-13]. We are focusing on identifying OBPs in antennae of *Anopheles gambiae*, because in Africa *A. gambiae* is the most important vector of *Plasmodium falciparum* [14], a major vector of *Wuchereria bancrofti*, which causes lymphatic filariasis [15], and a vector of O'nyong-nyong

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virus [16]. In this study, based on results of a screening microarray (unpublished) and previous microarray studies [9,11], we hypothesized that the OBP, OBP2 (AGAP003306), would have increased transcript accumulation by quantitative reverse transcription PCR (qRT-PCR) in female as compared to male *A. gambiae* antennae.

Methods

Collection and Processing of RNA

We studied AGAP003306, which had 2 fold greater expression in RNA isolated from antennae of 4 day old *A. gambiae* (Keele strain from Johns Hopkins) females than males in a microarray experiment (unpublished). In another microarray study of RNA from antennae of 5-7 day old Pink-eye *A. gambiae*, expression of AGAP003306 (OBP2) was 1.4 times higher in females as compared to males, but no qRT-PCR was done [9]. In yet another microarray study of RNA isolated from 3 day old whole mosquitoes (Pink-eye strain *A. gambiae*) there was approximately 3 fold increased expression in females vs males [17].

In this study, *A. gambiae sensu stricto* (G3 strain) were from the same batch of eggs (each batch giving rise to a mosquito lot) and were raised to adulthood under standard insectary conditions, and fed *ad libitum* with 10% sugar water [18]. We studied the antennae under controlled conditions of age and exposure to food. Adults of both sexes were collected exactly 4 days after emergence. The mosquitoes were immobilized by exposure to -20°C for 15 minutes, males and females separated, and antennae removed by manual dissection over dry ice, placed into separate 1.5 mL centrifuge tubes and homogenized using a pestle, each in 300 µL of Trizol reagent (Invitrogen, CA). RNA was isolated following manufacturer's instructions and purified using RNeasy mini column (Qiagen). The RNA was then assessed for quality and quantity using NanoDrop (ND-1000). The mosquito antennae that generated the RNA for the qRT-PCR experiments were isolated in July 2009 (mosquitoes from the University of Maryland), and January 2010 and June 2010 (mosquitoes from the National Institutes of Health).

qRT-PCR Assay

As an endogenous control, and foundation for the qRT-PCR analysis, we used the S7 ribosomal RNA gene of *A. gambiae* [19]. As another control we analyzed

AGAP009629, which did not have differential expression in antennae of females vs. males by microarray, but had increased expression in antennae of unfed vs. blood-fed females (unpublished).

The primer pairs synthesized and used (Table 1) were designed using Primer 3[20]. The QuantiTect SYBR Green RT-PCR kit (Qiagen) was used and reactions conducted in 96 well plates for 40 cycles (Applied Biosystems StepOnePlus™ System). Each reaction contained 12.5 µL Qiagen 2x Master Mix, 0.25 µL forward and reverse primers (0.5 µM final concentration), 0.25 µL RT Mix (containing reverse transcriptase), 10.75 µL DEPC water and 1.0 µL of RNA (0.05-1 µg/µL). The 96 well plate was sealed with adhesive film and centrifuged at 3700 rpm for 1 minute at 4°C. The ribosomal S7 gene was used as an endogenous control and a reaction without RT Mix (reverse transcriptase) was included for all reactions as a negative control. Changes in threshold cycles ($\Delta\Delta CT$) analysis was done to assess the ratio of RNA expression in females vs. males using previously published methods for analysis (Step One Software, v2.2, Applied Biosystems [21,22]).

Phylogenetic analysis

Multiple sequence alignments were built using the KALIGN program [23], followed by manual adjustments on the basis of profile-profile and structural alignments. Phylogenetic analysis was conducted using an approximately-maximum-likelihood method implemented in the FastTree 2.1 program under default parameters [24].

Results

qRT-PCR

RNA was extracted from paired antennae of approximately 100 female and 100 male *A. gambiae* derived from the same batch of eggs and raised in the same cage that had never been exposed to a blood meal. 80 ng RNA/mosquito to 560 ng RNA/mosquito (mean of 270 ng RNA/mosquito) was obtained. Approximately double the RNA was obtained from female as compared to male antennae. We isolated antennae on three separate occasions from three separate batches of mosquitoes over a period of 2 years. The results of three assays using RNA isolated from antennae (from three separate lots of mosquitoes) at three different times and normalized to the expression of the S7 ribosomal RNA gene are shown in Table 2. In all experiments there was an

Table 1 Primers used in qRT-PCR

AGAP ID	Forward primer	Reverse primer
AGAP003306	CTGCACATGGGCAAGCTG	CGTTCGCACACATCCTTG
AGAP009629	GCGTCCCAAGTTCAAAGTA	CGCAATGCACATCGTGTAG
Ribosomal S7	TGCGGCTTCAGATCCGAGTTC	TTCGTTGTGAACCCAATAAAAAATC

Table 2 Expression of AGAP003306 (OBP2) and AGAP0099629 (control) relative to expression of S7 in antennae of female and male *A. gambiae* in three biological replicates (experiments 1, 2 and 3).

Target	Experiment	Female			Male			Relative Expression	
		Ct Mean	Ct SD	ΔCt Mean	Ct Mean	Ct SD	ΔCt Mean	ΔΔCt	RQ
Reference S7	1	35.55	0.105		36.51	0.257			
	2	33.31	0.335		35.04	0.125			
	3	33.57	0.565		32.05	0.188			
AGAP003306	1	29.77	0.208	-5.780	33.87	0.414	-2.634	-3.145	8.849
	2	29.77	0.168	-3.540	33.87	0.414	-1.170	-2.370	5.171
	3	30.85	0.091	-2.721	33.99	0.309	1.941	-4.662	25.309
AGAP009629	1	35.42	0.298	-0.130	36.06	0.353	-0.450	0.320	0.801
	2	35.55	0.105	2.239	36.56	0.390	1.518	0.721	0.607
	3	36.46	0.478	2.894	34.95	0.686	2.894	0.000	1.000

Ct = (cycle threshold) number of cycles required for the fluorescent signal to reach threshold

Ct Mean = Average Ct from triplicate values on same sample in same PCR reaction

Ct SD = Standard Deviation of triplicate Ct

ΔCt Mean = Ct Mean_{Target} - Ct Mean_{Reference} (target=AGAP003306 or AGAP009629; reference=S7)

ΔΔCt = ΔCt Mean_{Female} - ΔCt Mean_{Male}

RQ = Relative Quantification = $2^{-(\Delta\Delta Ct)}$

increase in accumulation of transcripts of AGAP003306 in female vs. male *A. gambiae* (5 to 25 fold). When these data were integrated (Figure 1), there was a statistically significant increase in transcript accumulation of AGAP003306 in females vs. males ($p = 0.037$, paired student's t test). Furthermore, there was no significant difference in transcript accumulation between females and males of the control gene, AGAP009629 ($p = 0.246$, paired student's t test).

The relative increased accumulation of OBP2 transcripts in females vs. males in the three experimental (biological) replicates varied (Table 2). The mosquito

antennae that generated the RNA for the qRT-PCR experiments were isolated in July 2009 and were from mosquitoes from the University of Maryland (experiment 1), and in January 2010 (experiment 2) and in June 2010 (experiment 3) both from the National Institutes of Health, Bethesda. Thus, the differences in relative transcript accumulation were likely due to biological variability in gene expression from mosquitoes from different laboratories studied at different times. To determine if this was the case rather than variability of the assay, we repeated the qRT-PCR assays using the RNA of experiments 2 and 3. These technical replicates

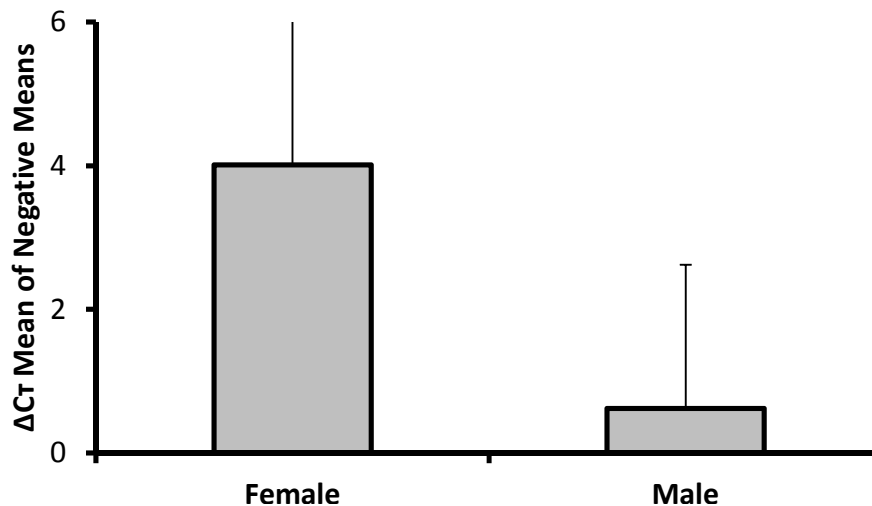


Figure 1 Comparative expression of AGAP003306 in female and male *A. gambiae*. Columns show mean of ΔCt Mean values (normalized amount of AGAP003306 RNA present) of the three experimental replicates shown in Table 1 multiplied by -1, error bars are standard errors. Data are significantly different in a pair student's t-test ($p = 0.037$, $n = 3$).

Table 3 Expression of AGAP003306 (OBP2) relative to expression of S7 in antennae of female and male *A. gambiae* in two technical replicates.

Target	Technical replicate	Female			Male			Relative Expression	
		Ct Mean	Ct SD	ΔCt Mean	Ct Mean	Ct SD	ΔCt Mean	ΔΔCt	RQ
Reference	Experiment 2	33.314	0.335		35.043	0.125			
		35.553	0.105		36.507	0.257			
S7	Experiment 3	33.565	0.565		32.053	0.188			
		36.502	0.309		31.034	0.400			
AGAP	Experiment 2	29.774	0.168	-3.540	33.873	0.414	-1.170	-2.371	5.172
		31.725	0.376	-3.828	34.759	0.191	-1.749	-2.079	4.226
003306	Experiment 3	30.845	0.091	-2.721	33.994	0.309	1.941	-4.662	25.309
		33.167	0.384	-3.335	32.711	0.562	1.678	-5.013	32.293

Ct = (cycle threshold) number of cycles required for the fluorescent signal to reach threshold

Ct Mean = Average Ct from triplicate values on same sample in same PCR reaction

Ct SD = Standard Deviation of triplicate Ct

ΔCt Mean = Ct MeanTarget- Ct MeanReference (target=AGAP003306 or AGAP009629; reference=S7)

ΔΔCt = ΔCt Mean^{Female} - ΔCt Mean^{Male}

RQ = Relative Quantification = $2^{-(\Delta\Delta Ct)}$

showed similar results as the original experiments (Table 3). In a recent publication, similar results for the OBP2 gene were found in a single biological replicate of RNA from 4-6 day old *A. gambiae* by RNA-seq [13].

Phylogenetic Analysis

OBP2 belongs to an OBP super family that includes the insect pheromone binding proteins [6]. Another member of this family, Agam OBP1, mediates indole recognition in antennae of female *A. gambiae* [7]. The olfactory receptors of terrestrial animals exist in an aqueous environment; yet detect odorants that are primarily hydrophobic. The aqueous solubility of hydrophobic odorants is thought to be greatly enhanced via OBPs, which exist in the extracellular fluid surrounding odorant receptors. This family includes proteins that specialize in binding insect pheromones (PBPs) and others that bind general odorants (GOBPs) [6]. Prior phylogenetic analysis has suggested that evolution of the OBP superfamily has evolved primarily through the process of lineage-specific expansion [25]. Thus, the majority of the OBPs in a given lineage such as Diptera, Hymenoptera, Lepidoptera or Coleoptera tend to cluster with others from the same lineage to the exclusion of those from other lineages. The genome of *A. gambiae* itself contains about 72 members of the OBP family.

We performed a phylogenetic analysis using over 100 representative OBPs from dipterans, hymenopterans and coleopterans with completely sequenced genomes. OBP2 is lodged within a predominantly dipteran lineage-specific expansion of OBPs that are particularly well represented in the culicid mosquitoes (Figure 2). This clade of OBPs includes the *Culex* (e.g. CquiOBP1) OBP that

binds the oviposition kairomones (5R, 6S)-6-acetoxy-5-hexadecanolide [26] (marked red, Figure 2). This analysis also showed that orthologs of *A. gambiae* OBP2 are conserved across *Culex*, *Aedes*, and *Anopheles* genera but are absent in *Drosophila* (blue box, Figure 2), pointing to a function for this protein in potentially binding a conserved odorant molecule in culicid mosquitoes. The up regulation of OBP2 observed in females as compared to males, suggests it could possibly bind a molecule comparable to the oviposition kairomone bound by OBP1. However, such a kairomone could also have an alternative role in guiding female feeding behavior. On the other hand it is also possible that OBP2 binds a pheromone that males express. The focus of future studies would be to pinpoint the role of this protein by determining the impact of knocking it down *vis-a-vis* feeding behavior and fitness of females.

New interventions are needed to control the mosquitoes that transmit the parasites that cause malaria [27,28] and lymphatic filariasis. Despite exciting scientific advances during the past few decades, no new approaches to mosquito vector control have been translated into widely used effective interventions. Sequencing the *A. gambiae* genome [29] and transcriptomics have provided a foundation for an approach to developing new interventions based on identifying genes and gene products that are important in transmission and mate-seeking. Stable genetic knockouts have not been generated in *A. gambiae*. However, transient knockdown by injection of sRNAi can be done and used to confirm the functional importance of OBP2 and other genes. This will be one of the next steps in our work.

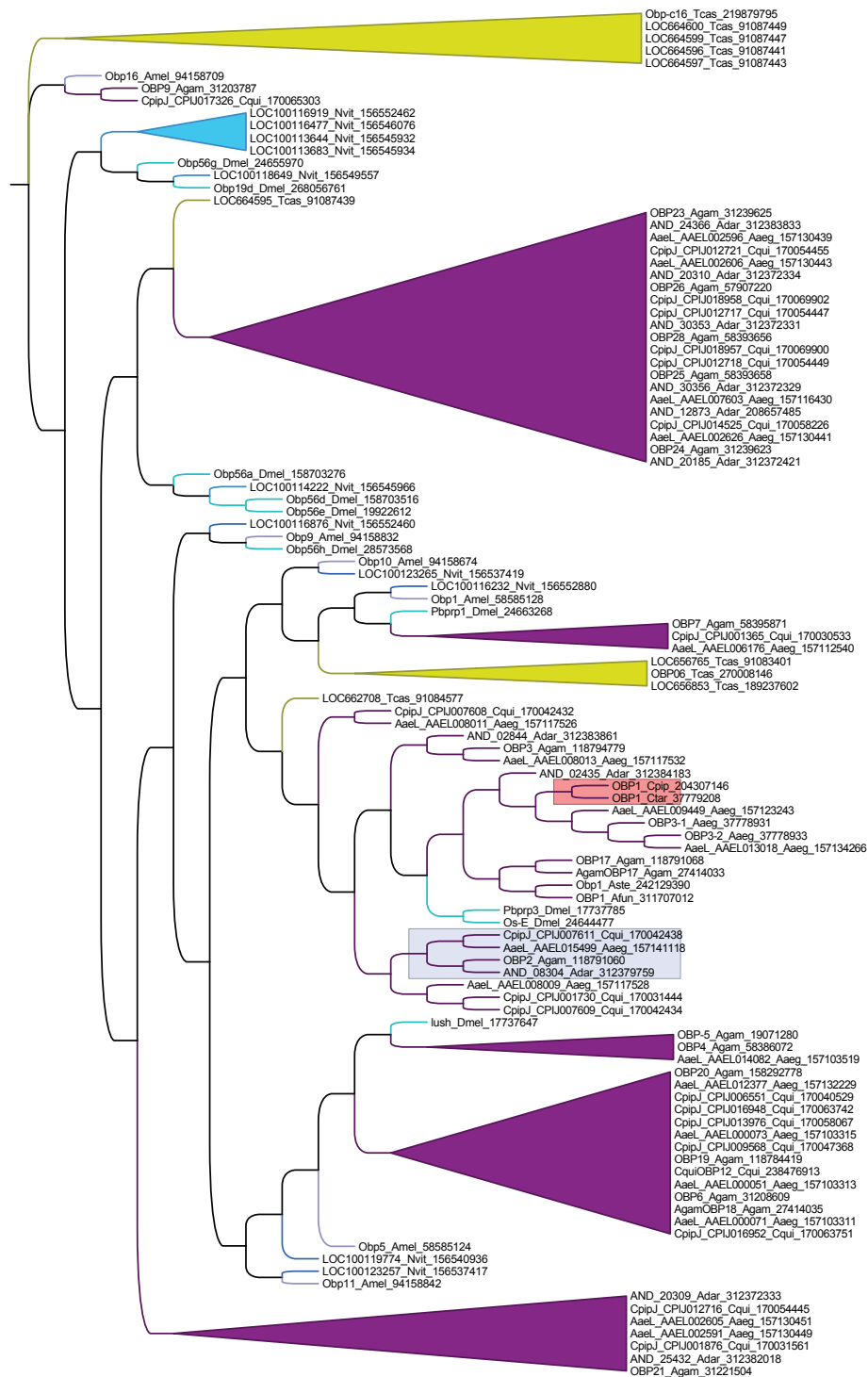


Figure 2 Phylogenetic analysis. Phylogenetic analysis was done using over 100 representative OBPs from dipterans (magenta), hymenopterans (turquoise) and coleopterans (yellow). The light blue box shows that orthologs of *A. gambiae* OBP2 are conserved across *Culex*, *Aedes*, and *Anopheles* genera, but are absent in *Drosophila*. The orange box shows that this clade of OBPs includes the *Culex* (e.g. CquiOBP1) OBP that binds the oviposition kairomones (5R, 6S)-6-acetoxy-5-hexadecanolide. The lineage-specific expansions in various insect lineages other than the OBP1 and OBP2 clades, which are discussed in the paper have been collapsed and the branch lengths equalized for simplicity of viewing.

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Authors' contributions

SAH: produced the preliminary data on which this work was based, developed the hypothesis and goals for the work, organized mosquito production, and conducted all other aspects of the work. These included dissections of antennae, RNA extractions, qRT-PCR, data analysis, preparation of figures, discussions and manuscript writing; LA: conducted the phylogenetic analysis of the data, prepared figures, and participated in discussions and manuscript writing; SV: was the senior investigator. He established the overall research framework, analysed data, prepared figures, and participated in discussions and manuscript writing. All authors read and approved the final version of the manuscript.

Competing interests

SV is an employee of Sanaria, Inc. Sanaria has no financial interest nor gain, nor competing interest with regard to this manuscript.

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