Odorant-Binding Proteins of the Malaria Mosquito
*Anopheles funestus sensu stricto*

Wei Xu, Anthony J. Cornel, Walter S. Leal

Department of Entomology, University of California Davis, Davis, California, United States of America

**Abstract**

**Background:** The mosquito *Anopheles funestus* is one of the major malaria vector species in sub-Saharan Africa. Olfaction is essential in guiding mosquito behaviors. Odorant-binding proteins (OBPs) are highly expressed in insect olfactory tissues and involved in the first step of odorant reception. An improved understanding of the function of malaria mosquito OBPs may contribute to identifying new attractants/repellents and assist in the development of more efficient and environmentally friendly mosquito controlling strategies.

**Methodology:** In this study, a large screening of over 50 ecologically significant odorant compounds led to the identification of 12 ligands that elicit significant electroantennographic (EAG) responses from *A. funestus* female antennae. To compare the absolute efficiency/potency of these chemicals, corrections were made for differences in volatility by determining the exact amount in a stimulus puff. Fourteen AfunOBP genes were cloned and their expression patterns were analyzed. AfunOBP1, 3, 7, 20 and 66 showed olfactory tissue specificity by reverse transcriptase PCR (RT-PCR). Quantitative real-time PCR (qRT-PCR) analysis showed that among olfactory-specific OBPs, AfunOBP1 and 3 are the most enriched OBPs in female antennae. Binding assay experiments showed that at pH 7, AfunOBP1 significantly binds to 2-undecanone, nonyl acetate, octyl acetate and 1-octen-3-ol but AfunOBP3, which shares 68% identity with AfunOBP1 at amino acid level, showed nearly no binding activity to the selected 12 EAG-active odorant compounds.

**Conclusion:** This work presents for the first time a study on the odorants and OBPs of the malaria vector mosquito *A. funestus*, which may provide insight into the *A. funestus* olfactory research, assist in a comparative study between major malaria mosquitoes *A. gambiae* and *A. funestus* olfactory system, and help developing new mosquito control strategies to reduce malaria transmission.

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**Introduction**

Malaria, the most serious mosquito-borne infectious disease, is widely distributed in the tropical and subtropical regions of the world. According to the last annual worldwide malaria morbidity and mortality survey in 2006, there were about 247 million cases among 3.3 billion people at risk, causing almost a million deaths, mostly in children under 5 years [1]. The principal malaria vector species in sub-Saharan Africa are *Anopheles gambiae sensu stricto* and *Anopheles funestus sensu stricto* [2,3], which belong to the same subgenus *Cellia* and diverged from a common ancestor approximately 5 million years ago [4]. Indeed, despite differences in morphology, breeding site preferences, mating behavior and relative seasonal abundance, both species coexist geographically in many parts of sub-Saharan Africa and both are highly anthropophilic and endophilic [2,3].

Despite extensive research conducted on the behavior, ecology and genomics of *A. gambiae*, much less is known about *A. funestus* since until very recently colonies of this latter species were unavailable. Vector control strategies must also impact and reduce *A. funestus s.s.* populations to reduce overall malaria incidence. In 2002, the genome project of *A. gambiae s.s.* was completed thus providing an invaluable resource to conduct comparative genetic and phenotype association studies among several *Anopheles* malaria vectors [5]. Many of these association studies include mechanisms of phenotypes that affect vector capacities of these malaria mosquito species, which will create new avenues for vector control strategies. Novel control strategies are needed as resistance to currently used pyrethroid insecticides have been recorded in both *A. gambiae* [6] and *A. funestus* [7].

Olfaction is essential in guiding insect behaviors such as foraging, host-seeking, and oviposition [8]. In female mosquitoes a population of hair-like sensilla distributed over the surface of the antennae and maxillary palps act as a nose to detect chemical signals. The major proteins involved in the selectivity and sensitivity of the insect olfactory system are odorant-binding proteins (OBPs) [9,10] and odorant receptors (ORs) [11]. OBPs are involved in the first step of odorant reception where they bind,
solubilize and deliver odorant molecules to ORs [10]. ORs are heterodimers comprised of highly variable odorant-binding subunits associated with one conserved co-receptor (OR39b) and localized on the dendrite membrane in the olfactory sensilla [12,13]. They detect odorant compounds and transduce olfactory signals to the brain to mediate insect behaviors.

The first insect OBP was discovered at the beginning of 1990s in the giant moth *Antheraea polyphemus* [9] while the first mosquito OBP (CquiOBP1) was isolated from antennae of female *Culex quinquefasciatus* in 2002 [14]. The release of the genome sequences of several mosquito species such as *An. gambiae*, *Aedes aegypti*, and *Cx. quinquefasciatus* has allowed the identification of large multigenic families of OBPs. To date, 33 classic OBPs have been identified in *An. gambiae* [15,16,17]; 34 classic OBPs were identified in *Ae. aegypti* [18], and 55 classic OBPs were identified in *Cx. quinquefasciatus* [19].

The literature documents various roles for insect OBPs. LUSH is a soluble OBP of the fruit fly *Drosophila melanogaster*. Deletion of LUSH gene suppresses *D. melanogaster* electrophysiological and behavioral response to the male pheromone 11-cis-vaccenyl acetate (cVA) [20]. Octanoic and hexanoic acids, two odorant compounds originate from the *Morinda citrifolia*, act as oviposition attractants for *D. sechellia* but as repellents for *D. melanogaster* [21]. Deleting OBP57d and OBP57e genes in *D. melanogaster* eliminates the avoidance behavior, while reinserting the orthologous genes of *D. sechellia* into *D. melanogaster* results in attraction to these two fatty acids [21]. *Bombyx mori* pheromone binding protein (BmorPBP1) and pheromone receptor BmOR1 were expressed in an "empty neuron" system of a *Drosophila* mutant and the response to the B. mori pheromone bombykol was analyzed [22]. Flies carrying both BmorPBP1 and BmOR1 showed significantly higher electrophysiological responses than flies carrying BmOR1 only [22]. Recently, two RNAi-mediated OBP genes silencing coupled with electrophysiological analyses have demonstrated the importance of OBPs in odorant recognition in two mosquito species [23,24]. By knocking down CquiOBP1 in *Cx. quinquefasciatus*, mosquitoes showed reduced antennal response to several oviposition attractants compared to controls [23]. Likewise, after injecting AgamOBP1 double-strand RNA into *An. gambiae*, mosquito response to indole was impaired [24]. All these studies showed that OBPs are critical for the selectivity and sensitivity of insect olfactory system. Therefore, the study on OBPs of malaria vector *An. funestus* might help us understanding the molecular basis of olfaction in this species and developing environmentally friendly strategies for mosquito control.

In this study, we have identified for the first time 12 odorants that elicited significant EAG responses from female *An. funestus* at 100 μg source dose (Fig. 1). By selecting 1-octen-3-ol as a standard, we identified octanal, nonanal, linalool, 2-undecanone, 2-heptanone, pentyl acetate, hexyl acetate, heptyl acetate, octyl acetate, nonyl acetate and ethyl hexanoate as the best ligands, i.e., compounds which elicited more than 50% of 1-octen-3-ol response. These 11 compounds, along with the standard 1-octen-3-ol, were further evaluated for their dose-dependent EAG response at 1, 10 and 100 μg source dose (Fig. 2). Chemicals screened in this study included compounds known to elicit significant antennal responses from *An. gambiae*, specifically formic acid, acetic acid, propanoic acid, lactic acid, p-cresol, 1-octen-3-ol, 7-octenonic acid, 3-methyl-2-hexenoic acid [25,26]. However, when tested on *An. funestus* these compounds, except 1-octen-3-ol, did not elicit significant EAG responses. This discrepancy between *Anopheles* species may be attributed to ecological differences between these two malarial vectors. Indeed, *An. funestus* differs markedly from *An. gambiae* in breeding site preferences, mating behavior and relative seasonal abundance [4].

1-Octen-3-ol was originally identified from the analysis of cattle odor [27] and later from human sweat [25]. It has been shown to serve as a powerful attractant for certain species of testes flies in the field [27]. Subsequent studies on mosquitoes have revealed that 1-octen-3-ol affects host-seeking behavior in mosquitoes [28,29,30]. Traps baited with 1-octen-3-ol resulted in moderate catch increase of a few mosquito species, but in combination with CO₂, 1-octen-3-ol serves as an efficient attractant [31]. Octanal and nonanal were both identified from human [32,33] and the latter has recently been shown as a major compound from both human and bird, which are alternate hosts of *Cx. quinquefasciatus* [33]. This compound elicited significant electrophysiology responses from female *Cx. quinquefasciatus* and nonanal baited traps added with CO₂ led to significantly higher catches of *Culex* mosquitoes [33]. 1-Octen-3-ol, octanal and nonanal were all identified from human, thus these compounds may be associated with the anthropophilic behaviors in *An. funestus*.

Interestingly, many floral and plant compounds viz., pentyl acetate, hexyl acetate, heptyl acetate, octyl acetate, nonyl acetate, ethyl hexanoate and 2-heptanone elicited high EAG activity in female *An. funestus*. These compounds may be involved in mosquito sugar-feeding behaviors. Sugar is the basic source of nutrients which provide energy to mosquitoes [34]. A ketone, 2-undecanone, was originally identified from wild tomato [35] and was later shown to have repellent activity against mosquitoes and ticks [36,37,38]. Linalool is a natural compound found in many flowers [39] and was also reported for its repellent activity on mosquito [40]. Thus, 2-undecanone and linalool that elicit high EAG responses in *An. funestus* may also be repellents for this mosquito species.

To overcome the difference in the volatility of test ligands during a stimulus [41], the amounts of chemicals released from the EAG syringe were quantified for the twelve compounds that elicited high EAG responses (Fig. 3A,B). The experiment showed that the amount of 2-undecanone released from the EAG syringe during stimulus was much less than the other chemicals as expected due to its larger molecular weight. To better correlate EAG response and the released amount of these odorants, we calculated the "EAG response/μmol" (Fig. 3C). The highest EAG response/μmol (mV/μmol) value was obtained for 2-undecanone.

**Results and Discussion**

**EAG study**

Our initial screening identified 12 odorants that elicited significant antennal responses from female *An. funestus* at 100 μg source dose (Fig. 1). By selecting 1-octen-3-ol as a standard, we identified octanal, nonanal, linalool, 2-undecanone, 2-heptanone, pentyl acetate, hexyl acetate, heptyl acetate, octyl acetate, nonyl acetate and ethyl hexanoate as the best ligands, i.e., compounds which elicited more than 50% of 1-octen-3-ol response. These 11 compounds, along with the standard 1-octen-3-ol, were further evaluated for their dose-dependent EAG response at 1, 10 and 100 μg source dose (Fig. 2). Chemicals screened in this study included compounds known to elicit significant antennal responses from *An. gambiae*, specifically formic acid, acetic acid, propanoic acid, lactic acid, p-cresol, 1-octen-3-ol, 7-octenonic acid, 3-methyl-2-hexenoic acid [25,26]. However, when tested on *An. funestus* these compounds, except 1-octen-3-ol, did not elicit significant EAG responses. This discrepancy between *Anopheles* species may be attributed to ecological differences between these two malarial vectors. Indeed, *An. funestus* differs markedly from *An. gambiae* in breeding site preferences, mating behavior and relative seasonal abundance [4].

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An arthropod repellent named BioUD, which was registered by US Environmental Protection Agency (EPA) in 2007, contains 7.75% of 2-undecanone. It was proposed to function as an effective alternative to DEET because of its repellent activity to mosquitoes [37].

Identification of fourteen putative OBP genes in *Anopheles funestus*

We have employed a homology cloning strategy based on previously identified *An. gambiae* OBP genes to isolate putative orthologs in *An. funestus*. We were able to clone fourteen genes,
AfunOBP1, AfunOBP3, AfunOBP5, AfunOBP6, AfunOBP7, AfunOBP9,
AfunOBP10, AfunOBP11, AfunOBP20, AfunOBP24, AfunOBP25,
AfunOBP26, AfunOBP28 and AfunOBP66 which display high
identity to their respective orthologs from An. gambiae. An
alignment of mature AfunOBPs amino acid sequences highlights
the high overall divergence of this family as only the six cysteine
residues are completely conserved between all proteins (Fig. 4).
Except for AfunOBP11 and AfunOBP29, the other AfunOBPs
share the characteristic features of the classic OBP family, namely,
small size, presence of a N-terminal signal peptide sequence as well
as a highly conserved pattern of six cysteine residues called the
“classic motif” [19] (Fig. 4). AfunOBP11 molecular weight is
about 21 kDa (Table 1) and it contains 12 cysteine residues,
whereas AfunOBP29 molecular weight is over 19 kDa and no
signal peptide was predicted. Additionally, both proteins do not
satisfy the classic motif of cysteine spacing [18] and Conserved
Domain Database (CDD) prediction also showed that these two
protein values for PBP/GOBP family are the lowest (Table 1) in all
14 AfunOBPs. However, their respective orthologous genes in
An. gambiae, AgamOBP11 and AgamOBP29, were classified as
“classic” OBPs in previous studies [17].

Each OBP from An. funestus belongs to different groups of
orthologous proteins previously described (Fig. 5) [19]. Two
proteins (AfunOBP1 and AfunOBP3) belong to the OS-E/OS-F
group, one (AfunOBP7) to the PBPRP1 group, one (AfunOBP5)
to the LUSH group, two (AfunOBP6 and AfunOBP20) to the
OBP19a group, and one (AfunOBP66) to the PBPRP4 group. All
the other 7 An. funestus OBPs are clustered into group B. Each

Figure 3. Relative EAG responses. (A) EAG responses (mV) of 12 selected chemicals at 100 µg source dose to the female An. funestus antennae
(n = 5; mean ± std); (B) amount of the odorants released from the EAG syringe during stimulus (n = 10; mean ± std); (C) EAG response per micro
molar of odorant (mV/µmol). The highest value on EAG response/µmol (mV/µmol) was recorded with 2-undecanone.
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Anopheles funestus Odorant Binding Proteins

AfunOBP is more closely related to putative orthologous OBPs in An. gambiae than to putative orthologous OBPs from other mosquito species (Table 2), as expected by An. funestus being more closely related to An. gambiae than to other mosquito species. Thirty-three “classic” OBPs have been identified from An. gambiae [15,16,17], thirty-four and fifty-three “classic” OBPs have been identified from Ae. aegypti [18] and Cx. quinquefasciatus [19], respectively. It is likely that more OBPs are present in An. funestus as the genome project of this species is not yet available and may provide more information about the complete repertoire of OBPs.

Expression profiles of An. funestus OBPs

Tissue specificity of fourteen An. funestus OBPs has been examined by RT-PCR in different tissues (olfactory tissues: antennae, maxillary palps, proboscis; non-olfactory tissues: legs and abdomen). Hitherto, most insect OBPs with a proven role in olfaction have been shown to be expressed exclusively in olfactory tissues. We hypothesize that an OBP gene abundantly and exclusively detected in chemosensory tissues likely encodes a “true” OBP [19]. In order to examine the transcripts levels between olfactory and non-factory tissues, amplification of a “house-keeping” gene encoding actin was used as control to check

Table 1. List of 14 cloned An. funestus OBPs parameters.

<table>
<thead>
<tr>
<th>OBP Name</th>
<th>GenBank accession #</th>
<th>Amino Acids</th>
<th>MW</th>
<th>pI</th>
<th>Cysteine Spacing</th>
<th>Signal Peptide %</th>
<th>CDD prediction (E-value)</th>
</tr>
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<tr>
<td>AfunOBP1</td>
<td>HM436669</td>
<td>144/125</td>
<td>14526</td>
<td>5.53</td>
<td>26/3/37/8/8</td>
<td>99.9</td>
<td>PBP_GOBP (1e_22)</td>
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<td>AfunOBP3</td>
<td>HM436670</td>
<td>151/124</td>
<td>14618</td>
<td>5.30</td>
<td>26/3/37/8/12</td>
<td>89.1</td>
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<td>13988</td>
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<td>26/3/41/10/8</td>
<td>98.6</td>
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<td>14287</td>
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<td>13/12/42/8/11</td>
<td>97.2</td>
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<td>139/122</td>
<td>14010</td>
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<td>35/29/3/33/8/15</td>
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Figure 4. Alignment of amino acid sequences of newly identified OBPs from An. funestus. Six conserved cysteine residues are highlighted in red.
doi:10.1371/journal.pone.0015403.g004
the integrity of each cDNA preparation. RT-PCR experiments showed that AfunOBP1, AfunOBP3, AfunOBP7 and AfunOBP20 were detected only from female antennae whereas AfunOBP66 was detected in both female antennae and proboscis (Fig. 6). On the other hand, remaining OBPs were detected in olfactory as well as non-olfactory tissues, indicating that they might not necessarily be involved specifically in olfaction.

We could not exclude that AfunOBP genes expressed in both olfactory tissues and non-olfactory tissues also function in the olfactory system. However, it is likely that olfactory-specific AfunOBP genes are directly involved in olfactory mechanisms and represent “true” OBPs. For example, CquiOBP1, which was only detected in Cx. quinquefasciatus olfactory tissues [19], was shown to bind to a mosquito oviposition pheromone (MOP), 6-acetoxy-5-hexadecanolid [42], in a pH-dependent manner [43]. By immunohistochemistry experiment with a specific antibody, this protein was detected in a subset of trichoid sensilla including one type responding to this pheromone but neither in the grooved peg sensilla nor in the basiconica sensilla on the maxillary palps [43]. By using RNA interference (RNAi), reduction of CquiOBP1 transcription in female antennae led to significantly lower electrophysiological responses to MOP and other known mosquito oviposition attractants [23]. AgamOBP1, which has significant high mRNA concentrations in female vs. male heads and is down-regulated after a blood meal, was suggested to be involved in female An. gambiae host-seeking behaviors [44]. Indole was identified as the only ligand with affinity to AgamOBP1 by using in silico as well as biochemical assays [24]. RNAi gene silencing coupled with electrophysiological analyses further revealed that An. gambiae response to indole was abolished after the knock-down of AgamOBP1 [24]. Interestingly, putative orthologous genes of olfactory-specific AfunOBP1, 3, 7, 20 and 66 were also detected at very high levels and/or exclusively in the olfactory tissues of An. gambiae [15] and Cx. quinquefasciatus [19] indicating that these proteins are likely involved specifically in chemoreception across several mosquito species.

Then, expression levels of olfactory-specific AfunOBP1, 3, 7, 20 and 66 were compared between female and male antennae by using quantitative real-time PCR (qPCR). Only female adult mosquitoes need a protein-rich blood meal to acquire nutrients necessary for eggs maturation after mating, while males do not feed on blood. Therefore, female specific or female enriched OBPs may be more specifically involved in host-seeking behavior. For such comparison, two different genes were used as endogenous control, actin and the OR63h-like odorant receptor cloned from An. funestus (AfunOR7).

Figure 5. Phylogenetic analysis of mosquito OBP amino acid sequences. 33 classic AgamOBPs (blue), 53 classic CquiOBPs (black), 34 classic AaegOBPs (purple), and new cloned 14 AfunOBPs (red) were grouped into OS-E/OS-F, PBPRP1, LUSH, OBP19a, PBPRP4, Group A and Group B. doi:10.1371/journal.pone.0015403.g005
When actin was used as control, female antennae over male antennae expression ratios (FA/MA) of all OBP genes ranged from 3.77 to 11.12, indicating a general enrichment in female antennae relatively to male antennae (Fig. 7A). Two genes, AfunOBP1 and AfunOBP3 displayed the highest enrichment in female antennae with FA/MA ratios of 7.89 and 11.12, respectively. AfunOBP7, 20 and 66 ratios displayed comparable but lower enrichment in female antennae, around 4 times. When AfunOR7 was used as control, FA/MA ratios were significantly reduced for all genes tested (Fig. 7B). Again, AfunOBP1 and AfunOBP3 displayed the highest enrichment in female antennae with FA/MA ratios of 3.22 and 4.54, respectively, and other genes (AfunOBP7, 20 and 66) displayed

<table>
<thead>
<tr>
<th>OBP</th>
<th>Phylogenetic group</th>
<th>An. gambiae homolog</th>
<th>Protein identity</th>
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<th>Protein identity</th>
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<td>AgamOBP1</td>
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<td>AaegOBP1</td>
<td>85%</td>
<td>CquiOBP1</td>
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<td>AfunOBP3</td>
<td>OS-E/OS-F</td>
<td>AgamOBP3</td>
<td>95%</td>
<td>AaegOBP38</td>
<td>90%</td>
<td>CquiOBP2</td>
<td>87%</td>
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<td>LUSH</td>
<td>AgamOBP5</td>
<td>91%</td>
<td>AaegOBP1/34</td>
<td>59%/54%</td>
<td>CquiOBP6</td>
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<td>AaegOBP22</td>
<td>70%</td>
<td>CquiOBP43</td>
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<td>AaegOBP10</td>
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<td>CquiOBP24</td>
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<td>AaegOBP40</td>
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<td>CquiOBP11</td>
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<td>87%</td>
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<td>79%</td>
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<td>60%/59%</td>
<td>CquiOBP14</td>
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![Figure 6. RT-PCR analysis of An. funestus OBPs in different tissues. An, antennae; Mp, maxillary palps; Pro, proboscis; L, Leg and Ab, abdomen. AfunOBP1, 3, 7 and 20 were detected only in the antennae while AfunOBP66 was detected in both antennae and proboscis. doi:10.1371/journal.pone.0015403.g006](image)
comparable but lower enrichment in female antennae, around 1.5–1.8 times. Expression analysis by qRT-PCR demonstrates that two antennae-specific OBP genes, AfunOBP1 and AfunOBP3, are both enriched in female antennae. Similar study on gender ratios (female/male) was performed on AgamOBP genes by microarray and qPCR [44]. AgamOBP1 and AgamOBP3, the orthologous genes of AfunOBP1 and AfunOBP3, were also detected at higher levels in female antennae than in male antennae [44]. By using microarray, AgamOBP3 showed a 9.2 times higher transcripts level in female antennae and AgamOBP1 showed a 4.2 times enrichment [44]. By using qPCR, AgamOBP3 showed a 8.1 times higher transcripts level in female head than in male and AgamOBP1 showed a 4.4 times enrichment [44]. Both approaches indicate that OBPI and OBPS are also female abundant OBPs in An. gambiae [44]. An AfunOBP1 orthologous gene from the Asia malaria vector Anopheles stephensi, AsteOBP1, was also cloned and studied recently [45]. The level of AsteOBP1 transcript was 7-fold higher in female antennae than in male antennae by qRT-PCR, revealing that AsteOBP1 was also abundant in female An. stephensi.

Sequence Analysis of AfunOBP1 and AfunOBP3

AfunOBP1 contains 144 amino acids. The 19 residues at the N-terminal were predicted as signal peptide (Table 1). The predicted mature AfunOBP1 showed 93% identity to AgamOBP1, 90% identity to CquiOBP1 and 85% identity to AaegOBP39. This OBP was initially isolated from antennae of the yellow fever mosquito, cloned, and named AaegOBP1 [46], but it was later renamed AaegOBP39 [18]. AfunOBP3 contains 151 amino acids. The 27 residues at the N-terminal were predicted as signal peptide (Table 1). The predicted mature AfunOBP3 showed 95% identity to AgamOBP3, 90% identity to AaegOBP38 and 87% identity to CquiOBP2. AfunOBP1 and AfunOBP3 shared 68% identity at amino acid level. Both of them belong to the OS-E/OS-F group (Fig. 5) and display the highest identity between orthologous proteins from different mosquito species (Table 2). Recombinant AfunOBP1 and AfunOBP3 were prepared by using a periplasmic expression system, which is known to generate properly folded, functional OBPs [47].

AgamOBP1, the orthologous OBP of AfunOBP1, undergoes a pH-dependent conformational change which is associated with a diminished capacity for binding [48]. The crystal structure of this protein suggests that the N- and C-termini of this protein may play a role in the reduction of binding by allowing these termini to unfold at low pH, thereby exposing the ligand to solvent [48]. The same phenomenon was also observed in the Ae. aegypti orthologous protein, AaegOBP1 (=AaegOBP39 [46,49]. Circular Dichroism

![Figure 7. Quantitative real-time PCR (qPCR) analysis of An. funestus OBPs from female and male antennae. Normalized by (A) actin gene and (B) AfunOR7. Error bars show standard deviation. Significantly differentially expressed AfunOBP genes for female and male antennae distributions were determined as p-value <0.05 by student t-test. doi:10.1371/journal.pone.0015403.g007](image-url)
(CD) and structural analysis indicated that AaegOBP1 (=Aae-
OBP39) undergoes a pH-dependent conformational change,
which may lead to release of odorant at low pH (as in the
environment in the vicinity of odorant receptors). A C-terminal
loop covers the binding cavity and this “lid” may be opened by
disruption of an array of acid-labile hydrogen bonds thus
explaining reduced or no binding affinity at low pH [49]. Based
on their high identity at the amino acid level, it is very likely that
AfunOBP1 also shares similar structure and pH-dependent
conformational change mechanism with its orthologous protein
in An. gambiae (AgamOBP1) and Ae. aegypti (AaegOBP1, later
named AaegOBP39 [49]).

Binding affinities of AfunOBP1 and AfunOBP3 towards
odorant ligands

Here fluorescence binding assay was used to determine the
binding activity of AfunOBP1 and AfunOBP3 to the EAG-active
compounds. Insect OBPs are involved in the first step of odorant
reception where they bind, solubilize and deliver odorant
molecules to ORs [10]. We hypothesized that AfunOBP1 and
AfunOBP3, two female most abundant olfactory tissue specific
OBPs in this study, show binding affinity to some or all of these
EAG active odorant compounds. The results showed that
AfunOBP1 displays high selectivity towards different EAG-active
ligands (Fig. 8A). The ligand with the highest affinity to AfunOBP1
was 2-undecanone, which is also the compound that elicited the
highest EAG response per μmol of odorant. 2-Undecanone was
first identified from the wild tomato [35] and later used as an
insect repellent on mosquitoes such as An. gambiae, Ae. aegypti
and ticks [36,37,38]. It is still unknown if 2-undecanone is also a
repellent to An. funestus. AfunOBP1 also showed binding affinity to
nonylacetate, octylacetate as well as 1-octen-3-ol. 1-Octen-3-ol
was identified from both cattle [27] and human [25] and has been
shown to serve as a powerful attractant for certain species of tsetse
flies in the field [27]. Traps with 1-octen-3-ol have resulted in
catches of only a few mosquito species, but in combination with

![Figure 8 Binding curves of EAG active compounds to AfunOBP1 and AfunOBP3. Error bars show standard deviation. At pH 7, AfunOBP1 (A) significantly binds to 2-undecanone, nonyl acetate, octyl acetate and 1-octen-3-ol but AfunOBP3 (B) showed almost no binding activity to any of the selected EAG active compounds.](doi:10.1371/journal.pone.0015403.g008)
CO₂, an increase in the collections has been observed [31]. There were no reports on nonyl acetate or octyl acetate to mosquito behavioral study yet. Both are compounds identified from fruits and may be involved in mosquito sugar-feeding behaviors [34]. CquiOBP1, AfunOBP1 orthologous OBP in Cx. quinquefasciatus, was successfully used as molecular target based on its binding affinity to identify several Culex mosquito oviposition attractants in a reverse chemical ecology approach [43]. This study indicated that AfunOBP1 may be used in screening strategies for potential attractants as well as repellents for An. funestus, which may help in mosquito control.

On the other hand, AfunOBP3, which shares high identity (68%) with AfunOBP1, showed no binding affinity to any EAG active compounds (Fig. 8B). Two highly similar mosquito OBPs showed very different characteristics in their binding affinities (Fig. 8), X-Ray crystallography and nuclear magnetic resonance (NMR) have been successfully applied to solve the structure of insect OBPs from Bombyx mori (BmorPBP1) [50,51], D. melanogaster (LUSH) [32], Leucophaea maderae (LmadPBP) [53], A. mellifera (Amel-ASP1) [54], A. polyphemus (ApolPBP1) [55], An. gambiae (AgamOBP1) [40] and A. aegypti (AaegOBP1) [49]. Comparative structural studies of AfunOBP1 and AfunOBP3 are certainly important in future research, particularly to address questions regarding molecular features of the pH-dependent conformational change observed for AfunOBP1. We have checked the effect of pH on the binding activity of AfunOBP1 to 2-undecanone (Fig. 9). AfunOBP1 showed high binding affinity to 2-undecanone at pH 7 but almost no binding affinity at pH 5. The same phenomenon was also observed on other insect OBPs. BmorPBP1 binds sex pheromone bombykol at pH 7 but not pH 5 [47,56]. CquiOBP1 bound MOP at high pH but not low pH. Several studies have suggested that the membrane surface around the dendrite in insect sensilla is negatively charged [57], which induces a drop in pH in the close vicinity of receptors. A pH-dependent ligand release mechanism is likely to also apply in AfunOBP1.

Conclusions
This work presents for the first time a study on the OBPs and odorants perceived by malaria mosquitoes An. funestus. By being highly anthropophilic and endophilic, An. funestus represents a very important malaria vector. Especially in recent years, it was suspected that due to the environmental changes or development of irrigation systems, favorable conditions have been created in Africa for the reestablishment of this dangerous species [58]. Here we performed a large EAG screening of over 50 ecologically significant odorant compounds and identified 12 ligands that elicit

Figure 9 pH-dependent binding of 2-undecanone to AfunOBP1. Binding curves of 2-undecanone to AfunOBP1 at pH 7 and pH 5 (A) and binding affinity of 2-undecanone to AfunOBP1 as indicated by replacement of a fluorescence reporter, NPN at pH 7 (B) and pH 5 (C) showed that AfunOBP1 can bind 2-undecanone at pH 7 but not pH 5. Error bars show standard deviation.
doi:10.1371/journal.pone.0015403.g009
significant antennal response from *An. funestus* female antennae, and may function as attractants or repellents for *An. funestus*. We also applied a molecular approach based on known genome information of three mosquito species, *An. gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus*, and successfully cloned 14 OBP genes from *An. funestus*. We demonstrated for the first time that two homologous OBPs, AfunOBP1 and AfunOBP3, sharing high identity at amino acid level and belonging to the same phylogenetic group (OS-E/OS-F), showed very different characteristics in their binding affinity. Binding assay experiments showed that AfunOBP1 significantly binds to 2-undecanone, nonyl acetate, octyl acetate and 1-octen-3-ol at pH 7 but AfunOBP3 showed nearly no binding activity to any of the selected odorant compounds at pH 7. These findings raise new questions: What changes on amino acid sequence cause these two AfunOBPs diverge in their characteristics in structures and binding affinities? What is the function of AfunOBP3? Addressing these questions will help us better understand the molecular mechanism and function of mosquito OBP genes. *An. gambiae* and *An. funestus* are both major malaria vector species in sub-Saharan Africa. If *An. conorensis* is excluded [59], *An. gambiae* s.s. is one member of a complex of 7 sibling species and *An. funestus* s.s. is one member of a group of 12 morphologically similar species some of which can be molecularly distinguished [60]. Members of the *An. gambiae* species complex and *An. funestus* species group, albeit being very closely related, have very different “biologies” that make some malaria vectors and others not. The interesting phenomenon to investigate is the genomic and biochemical parallels and differences that have evolved and allowed these two species to elicit such similar associations with humans. Both human blood feeding (anthropophily) and resting in human dwelling (endophily) behaviors are largely mediated by responses to chemicals in the environment. Comparative study on the olfactory systems of these two species will shed light on understanding the molecular mechanism that lead them to close association with humans and, therefore, could be of greatest interest in malaria control.

**Materials and Methods**

**Mosquito rearing**

*An. funestus* specimens used in this study were obtained from a sub colony established from the original FUMOZ colony [61]. Mosquitoes were maintained at 26°C, 85% RH with cycles of 12 hours light 10 hours dark with 1 hour dim light crepuscular periods in between to promote mating.

**EAG recording**

One to six days old non-blooded *An. funestus* females, fed on 10% sucrose *ad libitum*, were used. A head was excised with a sharp scalpel and placed on the reference electrode coated with electrode gel (Parker Laboratories, Orange, NJ). Distal end of the antennae (less than half a millimeter), cut to ensure a good electrical contact, were carefully placed on the recording electrode purchased from Syntech, Germany. EAG signals were fed to a 10× amplifier and processed with PC-based interface and software package (Syntech, Germany).

Preliminary experiment revealed that 1-octen-3-ol, a host derived kairomone identified from cows [27] and human sweat [25], elicits significant EAG response from female *An. funestus* antennae, as was also reported for *An. gambiae* [25,26]. Thus 1-octen-3-ol was used as a reference compound in screening of a wide array of chemicals that included alcohols: hexanol, heptanol, nonanol, 1-hepten-3-ol, 4-methylcyclohexanol, 3-octanol, (E)-2-hexen-1-ol, limonol and 1-dodecanol; aldehydes: hexanal, hepta-
CA) with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer's manual. PCR was performed in a GeneAmp PCR system 9700 thermal-cycler (Applied Biosystems, Foster City, CA) for 50 cycles with an annealing temperature of 55°C. PCR products were gel-purified using QIAquick gel extraction reagents (Qiagen, Valencia, CA), cloned into the pBluescript SK (+) cloning vector (Stratagene, Carlsbad, CA) and subsequently sequenced (http://www.davisssequencing.com/sample_prep.htm). 5'RACE (Rapid Amplification of cDNA Ends) PCR was performed according to SMART RACE cDNA amplification kit manual with universal primer and gene-specific primer (Table S2). 5'RACE PCR was carried out by using SMART RACE cDNA amplification kit and 5'-Full RACE Core Set (Takara, Madison, WI) with specific primers (Table S2 and S3). PCR products were further cloned into the pBluescript SK (+) cloning vector and sequenced. The complete AfunOBP nucleotide sequences have been deposited into Genbank under accession numbers given in Table 1.

Bioinformatics analysis of mosquito OBPs

N-terminal signal peptides of AfunOBPs were predicted by using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP). The calculated molecular weights and isoelectric points (pI) were obtained by using ExPaSy proteomics server (http://www.expasy.org/tools/protparam.html). Blast in NCBI conserved domains database (CDD) was used to identify PBP/GOB motifs. The amino acid sequence alignment of 14 AfunOBPs was performed by using clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). In this study, 14 new cloned AfunOBPs [13,16,17], 33 classic AgamOBPs [15,16,17], 34 classic AaegOBPs [18] and 53 classic CquiOBPs [19] amino acid sequences were used to create entry file for phylogenetic analysis in MEGA 4.0.2 [19]. An un-rooted consensus neighbor joining tree [62] was calculated at default settings with pair-wise gaps depletions as previously described [19].

Expression profiles of An. funestus OBPs

Total RNA was isolated from the 4–6 days old adult female antennae, maxillary palps, proboscis, legs and abdomens by using TRIzol and cDNA was synthesized from 1 μg of RNA as described above. All subsequent PCR reactions were carried out using 40 cycles with gene specific primers (Table S4). All RT–PCR reactions were replicated at least three times. Furthermore, the expression of cDNA End) PCR was performed according to SMART RACE cDNA amplification kit manual with universal primer and gene-specific primer (Table S2). 5'RACE PCR was carried out by using SMART RACE cDNA amplification kit and 5'-Full RACE Core Set (Takara, Madison, WI) with specific primers (Table S2 and S3). PCR products were further cloned into the pBluescript SK (+) cloning vector and sequenced. The complete AfunOBP nucleotide sequences have been deposited into Genbank under accession numbers given in Table 1.

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ABI 7300 Real Time PCR Instrument (Applied Biosystems, Foster City, California) was used with the Express SYBR @Green qPCR SuperMix Universal (Invitrogen) for quantitative real-time PCR. In each reaction, 10 μl of Supermix, 0.4 μl ROX Reference Dye (25 μM), 0.2 μl forward and reverse primers (Table S5), was added to 9 μl of a 96-well plate to which 1 μl of cDNA was added as a template. The cycling parameters were: 50°C for 2 min, 95°C for 2 min; 40 cycles of 95°C for 15s, 60°C for 1 min. For each cDNA sample and primer set, reactions were run in triplicate and average fluorescence Ct values were obtained. Relative gene expression ratios between female and male mosquitoes were determined using the Pfaffl method of analysis [63].

Protein expression, purification and circular dichroism analysis

pET22b vector was used for expression of recombinant AfunOBP1 and AfunOBP3 as described before [56]. Expression was performed in LB medium with transformed BL21 (DE3) cells (Novagen, San Diego, CA). Proteins in the periplasmic fraction were extracted with 10 mM Tris-HCl (pH 8) by using three cycles of freeze-and-thaw and centrifuging at 16,000 xg to remove debris. The supernatant was loaded on a Hisprep™ DEAE 16/10 column (GE Healthcare). Unless otherwise mentioned, all separations by ion-exchange chromatography were done with a linear gradient of 0–300 mM NaCl in 10 mM Tris-HCl (pH 8). Fractions containing the target protein were further purified on a 20 ml Q-Sepharose Hisprep™ 16/10 column (GE Healthcare) and, subsequently, on a Mono-Q HR 10/10 column (GE Healthcare). OBP fractions were concentrated by using Centriprep-10 (Millipore) and loaded on a Superdex-75 26/60 gel-filtration column (GE Healthcare) pre-equilibrated with 150 mM NaCl and 20 mM Tris-HCl (pH 8). Highly purified protein fractions were concentrated by Centricon-10, desalted on four 5-ml HiTrap desalting columns (GE Healthcare) in tandem and by using water as mobile phase, analyzed by LC-ESI/MS, lyophilized, and stored at −80°C until use. The concentrations of the recombinant proteins were measured by UV at 280 nm in 20 mM sodium phosphate (pH 6.5) and 6 M guanidine HCl by using the theoretical extinction coefficient calculated with EXPASY software (http://us.expasy.org/tools/protparam.html).

Fluorescence binding assay

N-phenyl-1-naphthylamine (1-NPN, also NPN) was used as a reporter ligand in fluorescence binding assay experiments. First the affinities of the 1-NPN to AfunOBP1 and AfunOBP3 were measured using 10 μg/ml protein solutions prepared in 20 mM ammonium acetate, pH 7. Increasing doses of 1-NPN (3.2 mM in ethanol, 0.5–2.5 μl) were added to the protein solutions until the fluorescence intensity reach a plateau. The amount of 1-NPN added was recorded and the fluorescence intensity was used as a reference (100%) to normalize the following measurements. For AfunOBP1 (10 μg/ml) at pH 7, 6.4 μM final concentration of 1-NPN was added to reach fluorescence intensity saturation while 3.2 μM final concentration of 1-NPN was added for AfunOBP3 (10 μg/ml) [Fig S1]. Then one of the selected EAG-active ligands was added (3.2 mM, 1–3 μl; final concentrations, 1.6–4.8 μM) and the fluorescence intensities were recorded and normalized by using the NPN reference. Fluorescence measurements were done on a spectrofluorometer (RF-5301, Shimadzu, Kyoto, Japan) at 25±1°C. Samples in 2-ml cell were excited at 337 nm, and the emission spectra were recorded from 350 to 500 nm, with emission and excitation slit widths of 1.5 and 10 nm, respectively [43].

Supporting Information

Table S1 List of primers designed for screening AfunOBP genes.
Found at: doi:10.1371/journal.pone.0015403.s001 (PDF)

Table S2 List of primers designed for cloning 3' and 5' RACE sequences of AfunOBP cDNA sequences. SMART RACE cDNA amplification kit was used.
Found at: doi:10.1371/journal.pone.0015403.s002 (PDF)

Table S3 List of primers designed cloning 5'RACE sequences of AfunOBP cDNA sequences. Takara 5'-Full RACE Core Set kit was used.
Found at: doi:10.1371/journal.pone.0015403.s003 (PDF)

Table S4 List of primers designed for AfunOBP genes RT-PCR analysis.
Found at: doi:10.1371/journal.pone.0015403.s004 (PDF)
Table S5  List of primers designed for AnfunOBP genes quantitative real-time PCR (qPCR) analysis.  
Found at: doi:10.1371/journal.pone.0015403.s005 (PDF)

Figure S1 Binding curves of 1-NPN to AnfunOBP1 and AnfunOBP3. To 10 μg/ml AnfunOBP1 (A) at pH 7, 3.2 μM of 1-NPN was needed to saturate the fluorescence intensity while fluorescence from 10 μg/ml of AnfunOBP3 (B) was saturated with 1.6 μM of 1-NPN.  
Found at: doi:10.1371/journal.pone.0015403.s006 (TIF)

References


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Author Contributions

Conceived and designed the experiments: WX AJC WSL. Performed the experiments: WX. Analyzed the data: WX AJC WSL. Contributed reagents/materials/analysis tools: WX AJC WSL. Wrote the paper: WX AJC WSL.


