

A synergistic transcriptional regulation of olfactory genes derives complex behavioral responses in the mosquito *Anopheles culicifacies*

Tanwee Das De^{1,2}, Tina Thomas¹, Sonia Verma¹, Deepak Singla¹, Charu Rawal¹, Vartika Srivastava¹, Punita Sharma¹, Seena Kumari¹, Sanjay Tevatiya¹, Jyoti Rani¹, Yasha Hasija², Kailash C Pandey^{1,3} and Rajnikant Dixit^{1*}.

¹ Laboratory of Host-Parasite Interaction Studies, National Institute of Malaria Research, Sector-8, Dwarka, Delhi 110077, India.

² Department of Biotechnology, Delhi Technological University, Shahbad Daulatpur, Main Bawana Road, Delhi-110042.

³ Department of Biochemistry, National Institute for Research in Environmental Health, Indian Council of Medical Research, Bhopal-462001

*Author for correspondence (dixit2k@yahoo.com)

Abstract: Decoding the molecular basis of host seeking and blood feeding behavioral evolution/adaptation in the adult female mosquito may provide an opportunity to design new molecular strategy to disrupt human-mosquito interactions. However, despite the great progress in the field of mosquito olfaction and chemo-detection, little is known that how the sex-specific specialization of the olfactory system enables adult female mosquitoes to derive and manage complex blood feeding associated behavioral responses. A comprehensive RNAseq analysis of prior and post blood meal olfactory system of *An. culicifacies* mosquito revealed that a minor but unique change in the nature and regulation of key olfactory genes play a pivotal role in managing diverse behavioral responses. Age dependent transcriptional profiling demonstrated that adult female mosquito's chemosensory system gradually learned and matured to drive the host-seeking and blood feeding behavior at the age of 5-6 days. A zeitgeber time scale expression analysis of Odorant Binding Proteins (OBPs) unravels unique association with a late evening to midnight peak biting time. Blood meal-induced switching of unique sets of OBP genes and Odorant Receptors (ORs) expression coincides with the change in the innate physiological status of the mosquitoes. Blood meal follows up experiments provide enough evidence that how a synergistic and concurrent action of OBPs-ORs may drive 'prior and post blood meal' complex behavioral events. Finally, tissue-specific gene expression analysis and molecular modelling predicted two uncharacterized novel sensory appendages proteins (SAP-1 & SAP2) unique to *An. culicifacies* mosquito and may play a central role in the host-seeking behavior.

Significance: Evolution and adaptation of blood feeding behavior not only favored the reproductive success of adult female mosquito but also make them an important disease vectors. Immediately after emergence, an environmental exposure may favor the broadly tuned olfactory system of mosquitoes to derive complex behavioral responses. But, how these olfactory derived genetic factors manage female specific 'pre and post' blood meal associated complex behavioral responses are not well known. We unraveled synergistic actions of olfactory factors governs an innate to prime learning strategy to facilitate rapid blood meal acquisition and downstream behavioral activities. A species-specific transcriptional profiling and an *in-silico* analysis predict novel 'sensory appendages protein', as a unique target to design disorientation strategy against the mosquito *Anopheles culicifacies*.

Introduction:

Mosquitoes are one of the deadliest animals in the world, which are responsible for transmitting a variety of infectious disease such as malaria, dengue fever, chikungunya, zika fever worldwide. According to WHO last annual report, malaria is one of the major vector-borne diseases that causes 212 million morbidity cases and more than 4 million mortalities (1). In India, malaria situation is more complex, where WHO states that the socio-economic burden of \$1.94 billion due to malaria alone (1). Current tools to control and manage malaria face challenges due to the emergence of parasite resistance to antimalarial drugs and insecticide resistance of mosquitoes (2), (3), (4), (5), (6), (7). Thus, alternative molecular tools are needed to rule out the expanding vector population as well as parasite development.

One of the key molecular strategies under not-to-bite approach relies on the designing of a new class of molecular tools able to disorient/alter the adult female mosquito host-seeking behavior (8). However, defining the molecular basis of host-seeking behavioral evolution and adaption to blood feeding by the adult female mosquitoes remains central to our understanding. Probably this may be due to the complex interaction of genetic and non-genetic environmental factors deriving mosquito navigation (9). In nature mosquitoes encounter many challenges to sustain in daily life i.e. they rely immensely on their sense of smell (olfaction) for the majority of their lifecycle stages (8). Mosquitoes are able to detect and discriminate thousands of odor molecules, which may further vary depending on the host preference. These complex behavioral events are largely mediated by the diverse chemosensory genes encoding odorant binding proteins (OBPs), odorant degrading enzymes (ODEs), odorant receptors (ORs) and other accessory proteins including sensory neuron membrane protein (SNMP) (10). Odorant binding proteins (OBPs), which are bathed within the sensillum lymph, are low molecular weight soluble proteins that mediate the first interaction of the olfactory system with the external world (10), (11), (12). These globular protein molecules showed significant diversity within the same family and are believed to bind with a wide range of hydrophobic odorant molecule. After binding with these odor molecules, OBPs shuttle it to their respective olfactory receptors present on olfactory receptor neurons (ORNs) (13), (10), (14). Insect olfactory receptors (OrX) are present to work in association with the obligate receptor co-receptor (Orco) on the dendritic membrane of ORN (10). Orco is essential for proper dendritic trafficking of the OrX, facilitating the formation of odorant gated ion channels by structural alteration that is opened upon odorant binding (10), (15). Thus, it is plausible to hypothesize that prior blood meal, key interactions of odorants and their cognate receptors may have a significant influence on food choice decision and blood meal uptake process.

For a successful blood feeding event, an adult female mosquito need to negotiate and manage multiple behavioral coordinates including searching, locating, landing over a suitable host, followed by tracing the proper site to pierce and suck the blood within two minutes (15), (16), (17), (18), (19), (20) (21). Just after the piercing organ (proboscis), it is the salivary glands which mediate the immediate biochemical interaction with the vertebrate blood and facilitate rapid blood meal uptake. Our recent study suggested that adult female mosquito's salivary glands are evolved with the unique ability of gene expression switching to manage meal specific (sugar vs blood) responses (22), but the molecular nature of the olfactory and neuro-system in regulating the salivary gland function is yet to unravel.

Post blood meal mosquitoes need to enter into a new habitat favouring successful oviposition (23), (24). In fact, after blood meal acquisition, mosquitoes undergo two major behavioral switching events (i) search suitable site(s) for temporary resting and completion of blood meal digestion (~30hrs) which is necessary for egg maturation (48-72hrs); and (ii) find proper oviposition site for successful egg laying (25). After completion of egg laying event, the adult female mosquitoes regain host-seeking behavioral activities for a second blood meal to complete the next gonotrophic cycles (24), (26). Notably, 'prior and post' blood meal associated habitats may have a significant difference in their physical, chemical and biological characteristics (23), but how olfactory driven factors manages these complex events is still an unresolved puzzle (27).

Immediately after emergence an exposure to diverse environmental/chemical cues facilitate olfactory maturation and learning for different innate behavioral activities such as feeding and mating in both the sexes (9). However, in case of adult female mosquitoes, we opined that the evolutionary forces, might have favoured an extra but unique specialization of host seeking and blood feeding behavioral

adaptation, possibly a result of frequent mosquito-vertebrate interactions (**Fig.1**). Once, a mosquito takes first blood meal it needs to manage major physiological activities linked to blood meal digestion and egg maturation. These physiological changes possibly may have another level of impact on olfactory responses to guide oviposition site finding behavior. We further hypothesize that first blood meal exposure must have a priming effect on the olfactory responses expediting the consecutive host seeking and blood feeding behavioral activities more rapidly than previous one.

To test and decode this evolutionary speciality, we performed RNAseq transcriptomic analysis of the olfactory system of adult female *An. culicifacies* mosquito, responsible for more than 65% malaria cases in India (28). A comprehensive molecular and functional annotation of RNAseq data unravelled a limited but remarkable change in the nature and regulation of unique sets of olfactory gene repertoire in response to distinct feeding status of the mosquitoes. Extensive transcriptional profiling of the selected transcripts showed biphasic and synergistic regulation under the distinct innate physiological status of the mosquitoes, possibly to facilitate and manage the complex host-seeking behavioral events. Finally, our structural bioinformatic analysis predicts the key residues of the selected sensory appendages proteins, targets for future functional validation and characterization.

Material and Methods:

Fig. 2 represents a technical overview of the current investigation.

Mosquito Rearing and Maintenance: A cyclic colony of the mosquito *An. culicifacies*, sibling species A and *An. stephensi* were reared and maintained at 28±2°C, RH=80% in the central insectary facility as mentioned previously (22), (29). All protocols for rearing and maintenance of the mosquito culture were approved by ethical committee of the institute.

RNA isolation and Transcriptome Sequencing Analysis: Complete olfactory tissue which includes antennae, maxillary palp, proboscis and labium, were dissected from 0-1 day of age, 30 min post blood fed and 30 hrs post blood fed *An. culicifacies* mosquito and collected in Trizol Reagent. Total RNA was isolated and double-stranded cDNA library was prepared by a well-established PCR-based protocol described previously (22), (30). Whole transcriptome sequencing of the olfactory tissue was performed by following the Illumina MiSeq 2 X 150 paired-end library preparation protocol. The sequencing data analysis pipeline is shown in Fig 2. A *denovo* clustering using CLC Genomics workbench (V6.2) (31) was used to build final contigs/transcripts dataset for functional annotation. For comprehensive annotation and differential gene expression analysis we followed essentially the same protocol as described earlier (22). Relative quantification of the genes was calculated by FPKM values (Fragments Per Kilobase of Exon Per Million Fragments Mapped).

Identification and molecular cataloguing of olfactory genes in *An. culicifacies*: An initial BLAST search analysis predicted a total of 93 transcripts encoding putative OBP homologs from the olfactory transcriptome data of *An. culicifacies* mosquito. To predict additional OBPs, a merged database of mosquito and *Drosophila* OBPs was re-queried against *An. culicifacies* draft genome/predicted transcripts databases available at www.vectorbase.org and build up the final OBP catalogue and classification for phylogenetic analysis as detailed in the supplemental **figure S1a**. A PDB database homology search analysis and GO annotation was used to identify and catalogue other putative olfactory receptor genes manually.

PCR based Gene Expression Analysis: The head tissue containing the olfactory appendages of female *An. culicifacies* mosquito was dissected at different zeitgeber time point. The 24 hr time scale of the LD cycle is represented as different Zeitgeber time (ZT) where ZT0 indicate the end of dawn transition, ZT11 is defined as the start of the dusk transition and ZT12 is defined as the time of lights off (32). Along with that, different tissues viz. head (male, female), Legs (male, female), brain,

olfactory tissue (OLF), female reproductive organ (FRO) and Male reproductive organ (MRO) of both *An. culicifacies* and *An. stephensi* mosquitoes were dissected and collected in Trizol followed by total RNA extraction and cDNA preparation. Differential gene expression analysis was performed using the normal RT-PCR and agarose gel electrophoresis protocol. For relative gene expression analysis, SYBR green qPCR (Thermo Scientific) master mix and Illumina Eco Real-Time PCR machine were used. PCR cycle parameters involved an initial denaturation at 95°C for 5 min, 40 cycles of 10 s at 95°C, 15 s at 52°C, and 22 s at 72°C. Fluorescence readings were taken at 72°C after each cycle. The final steps of PCR at 95°C for 15 sec followed by 55°C for 15 sec and again 95°C for 15 sec was completed before deriving a melting curve. To better evaluate the relative expression, each experiment was performed in three independent biological replicates. Actin or S7 gene were used as internal control in all the experiment and the relative quantification was analysed by $2^{-\Delta\Delta Ct}$ method (33). Differential gene expression was statistically analysed using student 't' test.

Blood meal time series follow up: For blood meal time series follow up the experiment, the olfactory tissues were collected from both naïve sugar fed and blood fed mosquitoes at different time points. Olfactory tissues collections were initiated from 0-1 day of naïve sugar-fed mosquitoes and proceed up to 6-7 days on every alternative day. After the 6th day, the adult female mosquitoes were offered first blood meal by offering a live animal (rabbit) and immediately collected olfactory tissues for 30 minutes' time point. The full blood-fed mosquitoes were separated and kept in a proper insectary condition for further experiment. After collection of olfactory Tissues at 30hrs and 72 hrs post blood fed the gravid females were kept for oviposition and again dissected OLF tissues after 24 hrs of the egg laying event. Second blood meal was provided to the egg laid mosquitoes and final collection of OLF tissues were done after 30hrs of 2nd blood meal.

Structural Modelling of SAP1 and SAP2: For structure prediction analysis of SAP1 and SAP2 proteins from *An. culicifacies*, initially, template for each query proteins was searched against PDB database using blastp algorithm. Two best templates were selected for each used query sequence and thereafter, modeller9 v.13 was used for the building of 50 models for each query sequence using multiple templates. The best model was selected based on the PROCHECK analysis, and DOPE score. Finally, the selected models were used for binding site prediction using COACH software.

Results and Discussion:

It is well known that a circadian dependent modulation of olfactory responses significantly influences the biting behavior in *Anopheline* mosquito species (32). But the knowledge that how this olfactory derived modulation manages pre and post blood meal behavioral events is yet not well understood (24), (25) (27). Based on available literature and knowledge gaps, we argued that immediately after emergence adult female mosquitoes must undergoes two unique changes in their olfactory responses i.e. (i) an exposure to the diverse chemical environment affecting host-seeking behavioral maturation that facilitate feeding preference switching from nectar to blood meal; (ii) blood meal digestion completion, enabling successful egg maturation and re-switching olfactory actions towards oviposition site finding behavior.

To decode and establish this molecular relationship managing 'prior and post' blood meal behavioral events we developed a working hypothesis (**Fig. 1**), a plausible mechanism which may have a significant influence on mosquito feeding and survival in diverse ecologies. To test the above hypothesis, first, we generated and analysed a total of ~122 million RNAseq reads of the olfactory tissues collected from 1-2 day old naïve (Nv), 5-6-day old immediate blood fed (30m-2h PBM) and 30hr post blood fed (30hr PBM) mosquitoes (Table-1). We chose 30h PBM as a critical time when completion of blood meal digestion occurs in the midgut, which may have direct influence on the reactivation of the olfactory system (**Fig. S2**) (24), (25), (34). For molecular and functional annotation, we assembled each transcriptomic database into contigs/Transcripts and compared against multiple molecular databases as described earlier (22). **Table-S1** represents details of the annotation kinetics of mosquito olfactory databases.

Blood meal causes modest but unique changes to olfactory responses: To test whether blood meal alters the global expression pattern of the olfactory transcriptome, we performed a digital gene expression analysis. Initial attempt of cleaned reads mapping to the available draft reference genome failed to yield quality results, probably due to poor annotation (**Fig. S3**). Alternatively, we mapped all the high quality reads against *Denovo* assembled reference map, as described earlier (22). Blood meal causes a modest shift in the transcriptome expression (**Fig. 3a**), supporting the previous report that first blood meal enhances odorant receptor transcripts abundance modestly, but causes general reduction of mosquito antennal chemosensory gene repertoire in *An. gambiae* (24).

We observed that at least >85% transcriptome remains unaltered, while only ~6% transcripts are up-regulated and ~8.7% transcripts downregulated in 30 min post blood fed samples (**Table-S2 and Dataset S1**). As expected ~10% transcripts expression was further reduced in 30h post blood fed olfactory tissue samples while only 2% transcripts up-regulated when compared to naive unfed mosquitoes (**Table S2**). Interestingly, a comprehensive annotation analysis also predicted that basic composition of the mosquito olfactory tissue does not alter significantly (**Fig. 3b-d**). Owing to a limited change in the olfactory responses, we hypothesize that blood-feeding may not directly cause a major shift in transcript abundance but may alter the functional nature/regulation of the unique transcripts controlling key biological processes such as response to stimulus, circadian rhythm, and signalling in the blood fed adult female mosquitoes. To clarify this complexity, we manually shortlisted the olfactory transcripts either based on their FPKM abundance and/or predicted coding nature and analysed a set of unique genes likely to influence mosquito host-seeking and blood-feeding behavior. To trace the possible molecular link, we extensively profiled their transcriptional regulation under distinct feeding status (see below).

Daily rhythm and expression change of Odorant binding proteins (OBPs) may influence olfactory responses: To negotiate and manage the navigation trajectory towards the vertebrate host, olfactory encoded odorant binding proteins (OBPs) play a crucial role to bind and deliver the odorants/chemicals to their cognate odorant receptors, an event guiding behavioral decisions. To explore the possible role of OBPs in the regulation of the olfactory behavior we identified and catalogued a total of sixty-three OBP genes from the mosquito *An. culicifacies* (**Table 1a**). Domain prediction analysis classified the OBPs as Classic OBPs, Plus-C OBPs, Two-domain OBPs and other Chemosensory protein family (**Table 1b; details in table S3**), as described earlier for the mosquito *An. gambiae* (35).

Table 1

(a)

SI No.	Sample Name	Number of OBPs Transcript Retrieved
1.	Ac-OLF-Naïve	14
2.	Ac-OLF-30min PBM	10
3.	Ac-OLF-30Hr PBM	12
4.	Ac-Genome Predicted	27
	Total	63

(b)

SI No.	Family of OBPs	Number
1.	Classic OBPs	26
2.	Plus-C OBPs	13
3.	Two-Domain OBPs	13
4.	Other Chemosensory Proteins	11

A comprehensive phylogenomic analysis of the Classic putative OBPs of *An. culicifacies* highlights the conserved sequence relationship with *An. gambiae* and other mosquito/insect species (**Fig S4a**), however, Plus-C and Atypical class of OBPs showed a distant relationship to the non-mosquito species *Drosophila* (**Fig. S4b, c**), probably evolved to facilitate host seeking and blood feeding behavior in adult female mosquitoes.

Interestingly, differential gene expression (DGE) data indicated that blood meal restricted the expression of common OBP transcripts (**Fig. S5**). However, first blood meal causes the appearance of unique OBP transcripts (**Table 1**), a crucial event in modulating the behavioral activities in response to changing the feeding status i.e. naive sugar to blood feeding. To further validate and unravel this unique relationship of OBPs regulation, we examined the expression of at least 15 putative OBP transcripts under distinct feeding status of the mosquitoes. In this analysis, we also included two uncharacterized chemosensory proteins (CSPs) named sensory appendage protein (SAP1 & SAP2) having a dominant expression in the naive mosquito olfactory tissue (**Table S3**).

Our Zeitgeber time scale expression showed that out of selected nine OBPs transcripts, at least 6 OBPs showed a >2fold modulation in their expression during late evening to midnight, in the 6-day old naïve mosquitoes (**Fig. 4a**). These data also corroborate with the previous observation of the natural active biting behavior of *An. culicifacies* mosquito in the mid night (36), (37). Surprisingly, sensory appendage proteins (Ac-SAP1 & Ac-SAP2) showed unequivocally an enriched [16 fold for SAP1, $p \leq 0.001$ and 6 fold SAP2, $p \leq 0.0001$] expression than other tested OBPs. Apart from that, we also observed a transient suppression (30min) and rapid recovery of OBPs expression just after a first blood meal (**Fig. 4b**). Together these observations suggested that a midnight hyper activities of OBPs are able to derive female specific host seeking behavioral activities of *An. culicifacies*. However, a transient change in expression in response to first blood meal further raises a question that how mosquitoes manage blood feeding associated complex behavioral responses such as egg maturation, oviposition etc. We hypothesize those harmonious actions of OBPs with ORs, which are involved in downstream odorant signal transduction cascade, may influence behavioral switching responses from naive sugar feeding status to blood feeding.

Innate physiological status may influence olfactory receptor responses to manage behavioral switching events: Current literature suggested that a combinatorial coding mechanism of the olfactory receptors enables insects to recognize thousands of diverse chemical cues for selective neuro-actions to meet specific behavioral demands (11), (14), (38). But, the molecular basis that how olfactory receptors superintend and co-ordinate between innate and primed/adaptive odor responses involving ‘prior and post’ blood meal behavioral complexity is yet to be unravelled.

After a successful blood meal, the gut physiology of the naive adult female mosquito undergoes a complex modulation to digest the blood meal and maturation of the eggs. Once the blood meal digestion completed, the mosquitoes may re-switch their olfactory responses for oviposition site finding behavior (24), (39), (40), (41). A transient modulation of OBPs expression in response to blood meal further prompted us to decode and establish its correlation with the olfactory receptors. To unravel this relationship, initially we retrieved, pooled and catalogued a total of 603 unique transcripts linked to response to stimulus and signalling (RTSS) categories (**Fig. 3b, c, d**), encoding diverse nature of proteins such as anion binding, nucleic acid binding, receptor activity, hydrolases and transferase activity (**Fig. 5a**). A comparative GO score distribution analysis predicted lower score hits for the blood-fed cohorts than naive mosquitoes (**Fig. 5a**). Surprisingly, out of 603 transcripts, we noticed only 110 transcripts common to all, while >100 transcripts remain uniquely associated with individual physiological conditions compared in the study (**Fig. 5b**). Together these data suggested that blood meal not only delimits the expression of many olfactory genes but also enriches the expression of many unique transcripts having similar functions.

Mosquito olfactory receptors are thought to play a central role to receive and communicate the chemical message to higher brain centre through olfactory receptor neurons for decision making events (10), (42), (43). To further clarify this molecular relation, initially, we catalogued 50 different chemosensory receptors (Table- 2) comprising odorant receptors (ORs); gustatory receptors (Grs)

and variant ionotropic receptors (IRs), which appeared predominantly in the naïve and blood fed cohorts of the RTSS category (Table: S4). Interestingly, a cluster of 19 different olfactory receptor genes was found to be expressed abundantly and exclusively in the naïve mosquito [Table- S4]. At the same time, we also observed that a distinct repertoire of chemosensory receptor genes uniquely appeared in the blood fed cohorts, but their number is much lower than the naïve mosquito (Table S4). Observation of the constitutive expression of Orco and few other ORs and Grs (totalling 10 transcripts) in all the experimental conditions highlighted the importance of Orco for the presentation of other receptors in the olfactory system. Together, these data suggested that an abundant expression of olfactory receptors in naïve mosquitoes may be essential to encounter and manage different conflicting behavioral demands when changing from naïve sugar feed status to blood fed.

Table 2:

Sl. No.	Sample Name	Number of Olfactory Receptors Transcripts
1.	Ac-OLF-Naive	32
2.	Ac-OLF-30min PBM	11
3.	Ac-OLF-30hr-PBM	7
	Total	50

Unlike OBPs, poor modulation of olfactory receptor gene expression under circadian rhythm (**Fig. 5c**) suggested a minimal role of ORs in the initialization of host-seeking behavioral activities. Alternatively, we also interpreted that ORs may not have direct biphasic regulation, but may influence a successful blood feeding event. To further corroborate with the above propositions and uncover the functional correlations of olfactory receptor responses, we monitored the transcriptional regulation of the selected OR transcripts in response to two consecutive blood meal series follow-up (44). An age-dependent enrichment of OR transcripts till 6th day of maturation in the sugar-fed mosquitoes suggested that naïve mosquitoes may express and attain a full spectrum of chemosensory genes expression to meet all the needs of their life cycle requirements i.e. host seeking and mate-finding behavioral response (**Fig. 5d**).

First blood meal of 6th-day old naïve mosquitoes initiates the suppression of almost all the olfactory receptor transcripts within 30 minutes of blood feeding, whose expression almost ceased to a basal level at 30 hrs post blood meal, except the slight up-regulation of two transcripts named OR42 and OR62 (**Fig. 5d**). Apparently, after 30 hrs PBM we observed a significant modulation of receptor gene expression which started enriching till 72 hrs of post first blood meal, a time window coincides with the successful completion of the oviposition event. However, we did not observe any significant change in the expression of the receptor transcripts in response to second blood meal (**Fig 5d**). These findings strongly suggested that first blood meal exposure to odorant receptors may have priming effect over host seeking behavioral activities, enabling mosquito for rapid blood meal uptake for consecutive gonotrophic cycles.

Blood meal response to other olfactory proteins: Encouragingly, the above data prompted us to test transcriptional profiling of few uncharacterized chemosensory class of olfactory proteins, identified from the transcriptomic data (**Table S5**). Transcripts encoding orphan receptor R21, scavenger receptor class B (SRCB), an uncharacterized Protein (XP_001959820) and Sensory neuron membrane protein (SNMP) showed a similar pattern of regulation, suggesting that a combination of all the receptor type represented in the olfactory tissue of *An. culicifacies* mosquito function concurrently in nature's aroma world and changed significantly prior and after the first blood meal as compared to the consecutive second blood meal (**Fig. 6a**). The involvement of G-proteins and related metabotropic signalling mechanism in the olfactory signal transduction of insects remain controversial. However, a rapid and consistent induction of adenylyl cyclase gene after 30m PBM (**Fig. 6b**), supporting the previous hypothesis that the synthesis of the secondary messenger, cAMP by adenylyl cyclase, facilitates odorant mediated signal transduction process which further influence downstream behavioral responses (10). Surprisingly, a finding of <1% of transcripts encoding putative immune proteins, suggested the maintenance of a basal level of sterility is essential for proper olfactory functions (**Fig. S6**).

Sensory appendages proteins as a unique target to *Anopheles culicifacies*: Two mosquito vector species are predominant in India viz. *An. stephensi* and *An. culicifacies*, the former is the urban vector and later is the rural one (28), (45). *An. culicifacies* have been reported predominantly as zoophilic in India, while *An. stephensi* exhibit predominantly anthropophilic behaviour (28), (46), (47). Even, when reared under the same environment at the central insectary facility, still they display a significant difference in their behavioral properties such as feeding, mating, biting preferences etc. (personal observation/ST-S5). Though the molecular basis of such biological variation is yet to unravel, but emerging evidence suggest a significant genetic difference exists among various Anopheline mosquito species, including *An. stephensi* and *An. culicifacies* (48), (49). Under laboratory investigation, we frequently observed that biological rhythm may have a significant influence on the biting and blood feeding behavior of *An. culicifacies*. Previously, several odorant binding proteins such as OBP20/OBP1/OBP7 have been characterized as a key molecular target in many *Anopheline* mosquitoes involved in host-seeking behavior (50), (51), (52), but remains unidentified from *An. culicifacies*.

Thus, to test whether any species-specific olfactory derived genetic factors have any differential regulation, influencing the behavioral responses, we compared the expression of at least 6 OBPs transcripts between two laboratories reared mosquito species *An. stephensi* and *An. culicifacies*. In this analysis we also included two novel SAP proteins, which showed a high induction than other OBPs in the olfactory system of the mosquito *An. culicifacies* at midnight (**Fig. 4a**). Surprisingly, a sex and tissue specific comparative transcriptional profiling of selected OBPs revealed a dominant expression of SAP1 ($p \leq 0.0003$)/SAP2 ($p \leq 0.0007$) in the legs of mosquito *An. culicifacies* (**Fig 7a, b**). Together these data indicated that *An. culicifacies* may draw an extra advantage of having more sensitive appendages, possibly to favour more active late night foraging behavior, than *An. stephensi*. Furthermore, a strong phylogenetic association of Ac-SAP1 protein within Anthropophilic mosquito clade (**Fig. 7c**), strongly suggested that sensory appendages proteins may have a crucial role to meet and manage the high host seeking behavioral activities, restricted to *An. culicifacies*.

Encouraging to the above finding, we carried out a 3D structure modelling analysis of Ac-SAP1 and Ac-SAP2, to predict the best possible conserved binding pockets for specific chemicals. In the absence of any available solved X-ray structure of reference SAP protein, we applied a template based comparative molecular modelling approach. An initial blast analysis identified two best templates in PDB database code for chemosensory protein 2GVS and 1KX8 with identity 47-56% and coverage >80%, favouring their suitability for structure prediction. Out of the 50 modelled 3D structures for each protein, DOPE score analysis resulted in the selection of model-49 and model-27 with score -11689.73, and -10989.75 for SAP1 and SAP2 respectively (**Fig. 7d and Fig. S7a**).

We validated the best-selected model using Procheck server for Ramachandran plot, showing a more than 95% allowable region, with no residue falling in the disallowed region of the plot (**Fig. 7e and Fig. S7b**). Based on the consensus, a best-fit ligand binding site prediction analysis within the selected models was scored by COACH server, which engages at least five different algorithms TM-SITE, S-SITE, COFACTOR, FIND-SITE, and ConCavity. Binding pocket for SAP1 and SAP2 identified eight consensus residues namely D36, E39, L40, K49, C52, Q59, Y91, and Y95 along with BDD (12-bromo-1-dodecanol) as a predicted ligand. Minimization of the steric clashes from the complex structures was done using Chimera software (**Fig. 7f and Fig. S7c**). Furthermore, selection of amino acid residues within 3 Å region of ligand molecule are I43 and Y95 of which Y95 is involved in H-bonding with BDD ligand. Similarly, in case of SAP2 protein, residue selection resulted in the identification of I43, D51, Q59, T63, Y95 residues of which D51 form H-bond with BDD ligand (**Fig. 7g and Fig. S7d**).

In our analysis we observed the presence of at least two conserved cysteine (CYS52 and CYS55) residues in the loop region of SAP1 and SAP2 proteins, which may likely involved in di-sulphide bond formation and stabilization of protein structure. Our analysis also showed that binding pocket forms a tunnel-like structure which is preferred by long aliphatic molecules. Presence of negatively charged aspartic and glutamic acid at both ends showed the preference for charged residue near the vicinity of ligand molecule. Moreover, the presence of conserved negatively charged aspartic acid

and polar tyrosine (TYR91 in SAP1 and TYR95 in SAP2) at one end of binding pocket suggested their role in ligand binding.

A synergetic action of OBPs/ORs may manage behavioral responses: Decoding the genetic relationship of sense of smell is central to design new molecular tools to disrupt mosquito-human interaction. We demonstrated that a synergistic and harmonious action of olfactory encoded unique factors govern the successful ‘prior and post’ blood feeding associated behavioral complexities. We concluded that a quick recovery of the actions of odorant binding proteins immediately after blood feeding, and delayed re-activation of olfactory receptor proteins after blood meal digestion completion are unique to manage diverse behavioral responses. We hypothesize that first blood meal exposure is enough for prime learning, satisfying the motivational search of mosquitoes for the completion of their gonotrophic cycles. Thus, it is plausible to propose that apart from the innate odor responses, adult female mosquitoes might took an advantage of prior odor (vertebrate) exposure, which leads an exclusive evolutionary specialty, allowing them to learn, experience and adapt as a fast blood feeder in nature (Fig. 8).

In summary, we decoded and established a possible functional correlation that how a coherent and smart actions of olfactory encoded factors enabled adult female mosquitoes to meet and manage the blood feeding associated complex behavioral activities (**Fig. 8**). Furthermore, targeting species-specific unique genes such as sensory appendages proteins may be crucial to design disorientation strategy against mosquito *An. culicifacies*, an important malarial vector in rural India.

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Authors contribution statement: Conceived and designed the experiments: TDD, RD; Performed the Experiments: TTD, TT, SV, DS, VS, PS, CR, SK, ST, JR; Analyzed the data: TDD, RD; YH, Contributed reagents/materials/analysis tools: YH, RD, KCP; Wrote the paper: TDD, RD, YH, KCP.

Competing interest statement: The authors declare no conflict of interest

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Figure Legends:

Fig. 1. Working Hypothesis to establish functional co-relation of the olfactory system under distinct feeding status: Mosquitoes Adult mosquitoes, just after emergence from pupae are exclusive sugar feeders and dependent on nectar sugar to acquire energy for flight activity. Exposure of the adult mosquitoes to the diverse aromatic environment facilitate their learning and maturation of the olfactory system which enable successful mating and host seeking behavioral activities. But the function of the olfactory system starts to diminish just after blood feeding and become ceased at least for 30hrs of post blood meal. Blood feeding initiates lots of physiological changes including blood meal digestion in the midgut and egg maturation in the ovary which consume lots of energy and thus mosquitoes manipulate the energy cost by shutting down the olfactory responses and preferred to take rest at a cool dark place. After 30 hrs of blood feeding the blood almost digested in the midgut and maturation of egg reached a threshold level which reinforce the mosquito to perform to next level of behavior. Thus, recovery/reactivation of the olfactory responses occur to find a suitable site for egg laying/oviposition. To capture these molecular snapshot and track the events, we collected olfactory tissues at three different physiological conditions for RNASeq analysis (Highlighted as red arrows), and coupled with gene expression study with more elaborated time and physiological state (highlighted with blue arrows). MG: Midgut; OV: Ovary. Mosquitoes each and every life cycle stages are tightly regulated by circadian (dawn & dusk) cycle (Background light dark color code).

Figure 2: A technical overview to decode the hard-wired genetic structure of olfactory system of *Anopheles culicifacies*. We sequenced and analysed three RNA samples extracted from the olfactory tissues of approximately 30 mosquitoes individually and pooled to form one single sample. With this strategy we were able to quantify and validate the estimation of gene expression, expected with a minimum chance of aberrations as described earlier (21).

Figure 3: Blood meal cause modest changes in the molecular architecture of the mosquito olfactory system: (A) Read density map of the compared Naive; 30M and 30Hr post blood meal (PBM) transcriptomic data of olfactory system; (B-D) Functional annotation and molecular catalogue of olfactory transcriptome (Biological Process/Level2/% Transcripts). Red circle marks the genes selected for transcriptional response monitoring. (See Text).

Figure 4: Transcriptional profiling of the odorant binding protein genes (OBPs) under different circumstances. (a) Rhythmic expression of OBP genes in the adult female's olfactory tissues (OLF) according to different zeitgeber time (ZT) scale, where ZT0 indicate the end of dawn transition, ZT11 is defined as the start of the dusk transition and ZT12 is defined as the time of lights off. (b) Relative expression profiling of OBP genes in pre and post blood fed olfactory tissues. Olfactory tissues (OLF) were collected from 1day, 3day and 6day old sugar fed mosquitoes which were then provided blood meal and then the olfactory tissues were collected after 30mins of post blood fed and 30hrs and 72hrs of post blood fed mosquitoes. The significance of suppression of OBP genes expression after 30hrs of post blood meal are as follows: SAP – ≤ 0.004 ; SAP2 – ≤ 0.039 ; OBP7 – ≤ 0.007 ; OBP20 – ≤ 0.0004 ; OBP10 – ≤ 0.003 .

Figure 5: Blood meal modulates odorant receptors expression: a) A comparative GO score distribution analysis of response to stimulus and signalling transcripts of naïve and blood fed mosquitoes. b) Venn diagram showing common and unique transcripts of response to stimulus and signalling GO category of naïve and blood fed mosquitoes. (c) Rhythmic expression of olfactory receptor genes (Ors) of *An. culicifacies* in the olfactory tissues of female mosquitoes, where ZT0 indicate the end of dawn transition, ZT11 is defined as the start of the dusk transition and ZT12 is

defined as the time of lights off. (d) Transcriptional response of olfactory receptor genes according to blood meal time series experiment. Olfactory tissues (OLF) were collected from naïve sugar fed adult female mosquitoes till 6th day (OLF-1D, OLF-3D, OLF-6D). Then mosquitoes were provided blood meal and again olfactory tissues were collected at different time point after blood feeding, viz. OLF-30M: 30 min post blood fed (PBM); 30hr-PBM: 30hrs of PBM; 72Hr-PBM: 72hrs of post blood meal; then the mosquitoes were kept for oviposition (egg laying), and again the olfactory tissues were collected 24hrs of post oviposition (24hr-POT). Finally, the 2nd blood meals were provided to the egg laid mosquitoes and collected olfactory tissue 30hrs of second blood meal (30hr-PBM2). The significance of suppression of OR genes expression after 30hrs of post blood meal are as follows: Putative Or - ≤ 0.001 ; Or42 - ≤ 0.05 ; GR - ≤ 0.003 ; Or44 - ≤ 0.0002 ; IR41c - $\leq 3.5E^{-05}$; Or62 - ≤ 0.06 ; Or39 - ≤ 0.02 ; Or9 - NS.

Figure 6: Transcriptional responses of other olfactory genes hypothesized to play crucial role in host seeking and blood feeding behavior. (a) Relative expression profiling of other receptor genes according to blood meal time series (described in fig. 5). Orphan R21: Orphan receptor 21; Uncharacterized Pr: Uncharacterized protein; SRCB: Scavenger Receptor class B; SNMP: Sensory neuron membrane protein. (b) Transcriptional profiling of other signalling molecule in response to blood meal time series experiment. Circadian: Circadian gene; AC: Adenylyl cyclase. The significance of suppression of other olfactory genes expression after 30hrs of post blood meal are as follows: Orphan R21 - ≤ 0.002 ; Uncharacterized pr - ≤ 0.002 ; SRCB - ≤ 0.006 ; SNMP - ≤ 0.007 .

Figure 7: Comparative transcriptional responses of Odorant binding protein genes between two major Indian vectors and structural characterization of one of the potent OBP gene. (a, b) Sex and tissue specific relative expression profiling of OBP genes in *An. culicifacies* (a) and *An. stephensi* (b). FOLF: female olfactory tissue (OLF); MOLF: Male OLF; FRO: Female reproductive organ; MRO: Male reproductive organ; FLeg: Female legs; MLeg: Male legs. OBP gene details: SAP: Sensory appendages protein 1; SAP2: Sensory appendages protein 2. (c) Phylogenetic analysis of *An. culicifacies* SAP1 (Ac-SAP1) gene. (d) DOPE score analysis for SAP1. (e) Ramachandran Plot of SAP1 protein. (f) 3-dimensional protein structure of Ac-SAP1 protein. (g) Binding site of SAP1 protein showed in space fill with nearby residues in stick form.

Figure 8: How smart actions of olfactory system manages blood feeding associated odor response: an evolutionary speciality of adult female mosquitoes. After emergence from pupae adult mosquitoes are exposed to the overwhelmed odor world, where odorants chemicals act as a language of communication with the external world. The sophisticated innate olfactory system of mosquitoes enables them to recognize and differentiate these wide variety of odorants which are crucial for their every life cycle stages. Inner physiological motivation as well as the age and exposure of mosquitoes towards the external world promote them for host seeking and blood feeding event. After taking blood meal mosquitoes initiate next level of physiological cum behavioral events i.e. oviposition. Apart from that, first exposure with vertebrates facilitate learning and second blood feeding events. These whole odors mediated response is tactfully managed by the synergistic actions of Odorant binding proteins (OBPs) and olfactory receptors (Ors). The overlapping circadian rhythm dependent functions of OBPs and Ors govern the pre blood meal events of host fetching events. As soon as the mosquitoes take blood meal the functions of OBPs and Ors ceased for some period, but the recovery of OBPs actions occurs early as compared to Ors to perform the next level of behaviors. Mosquitoes, then take advantage/adapted from priming and learning of the first blood meal exposure for the more rapid consecutive blood feeding.

Figure files

Figure 1:

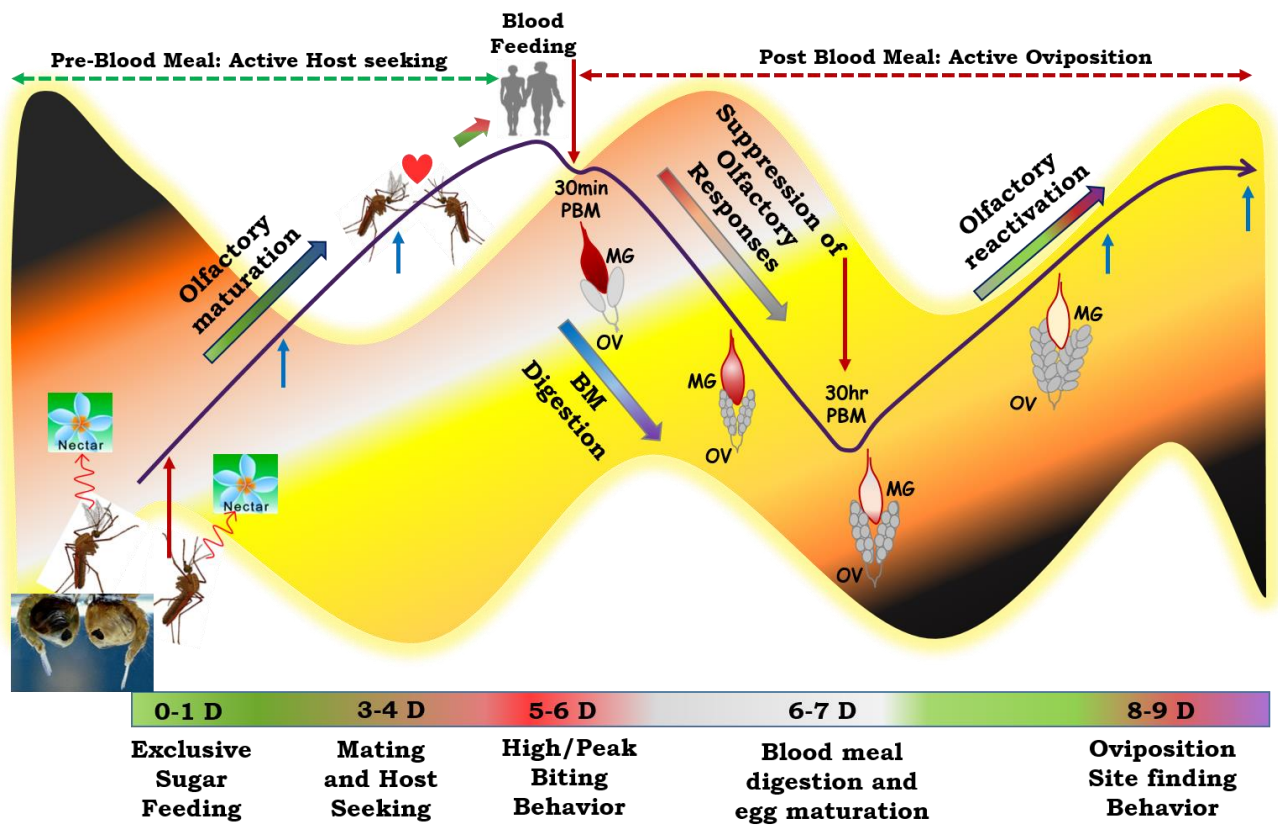


Figure 2:

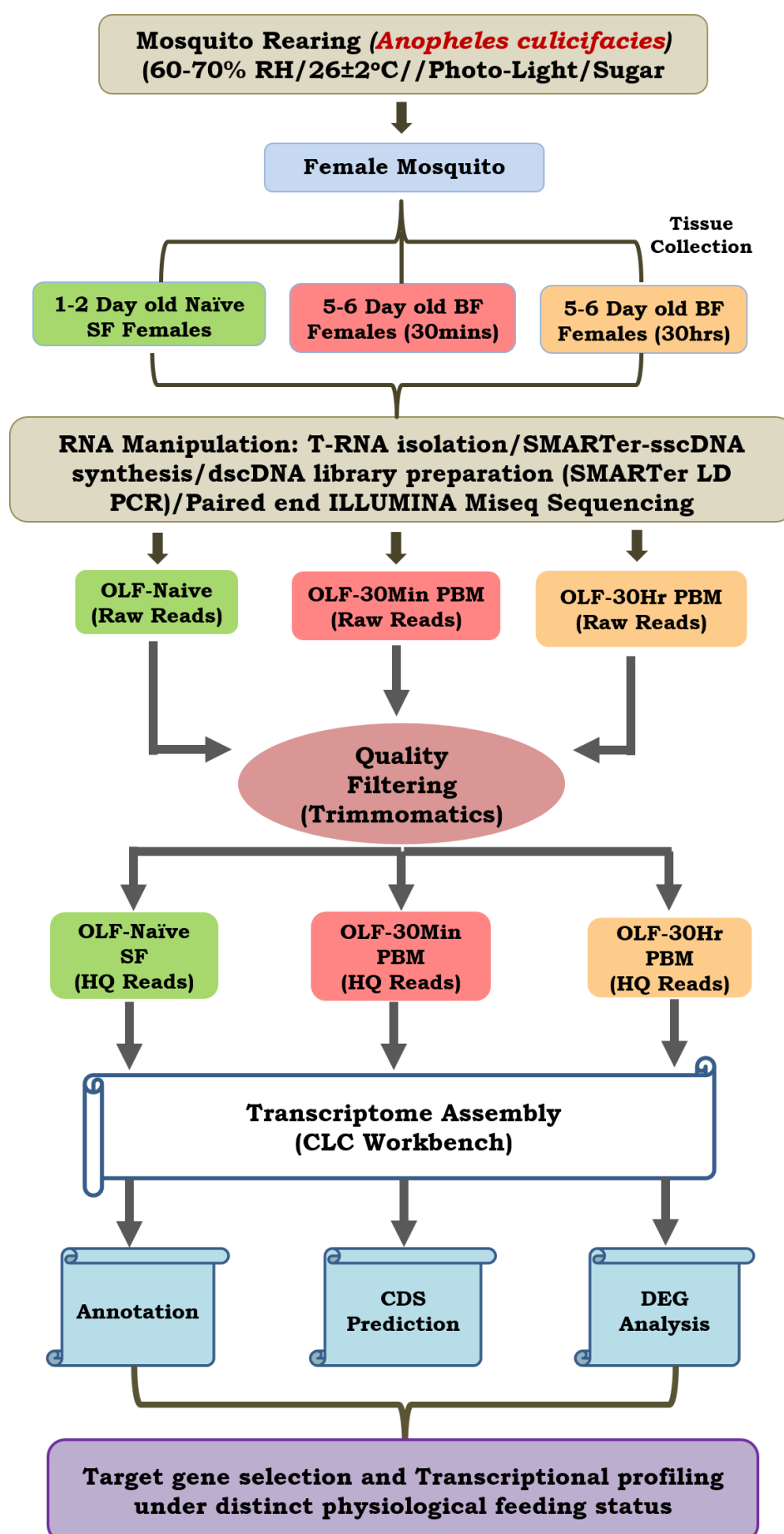


Fig 3

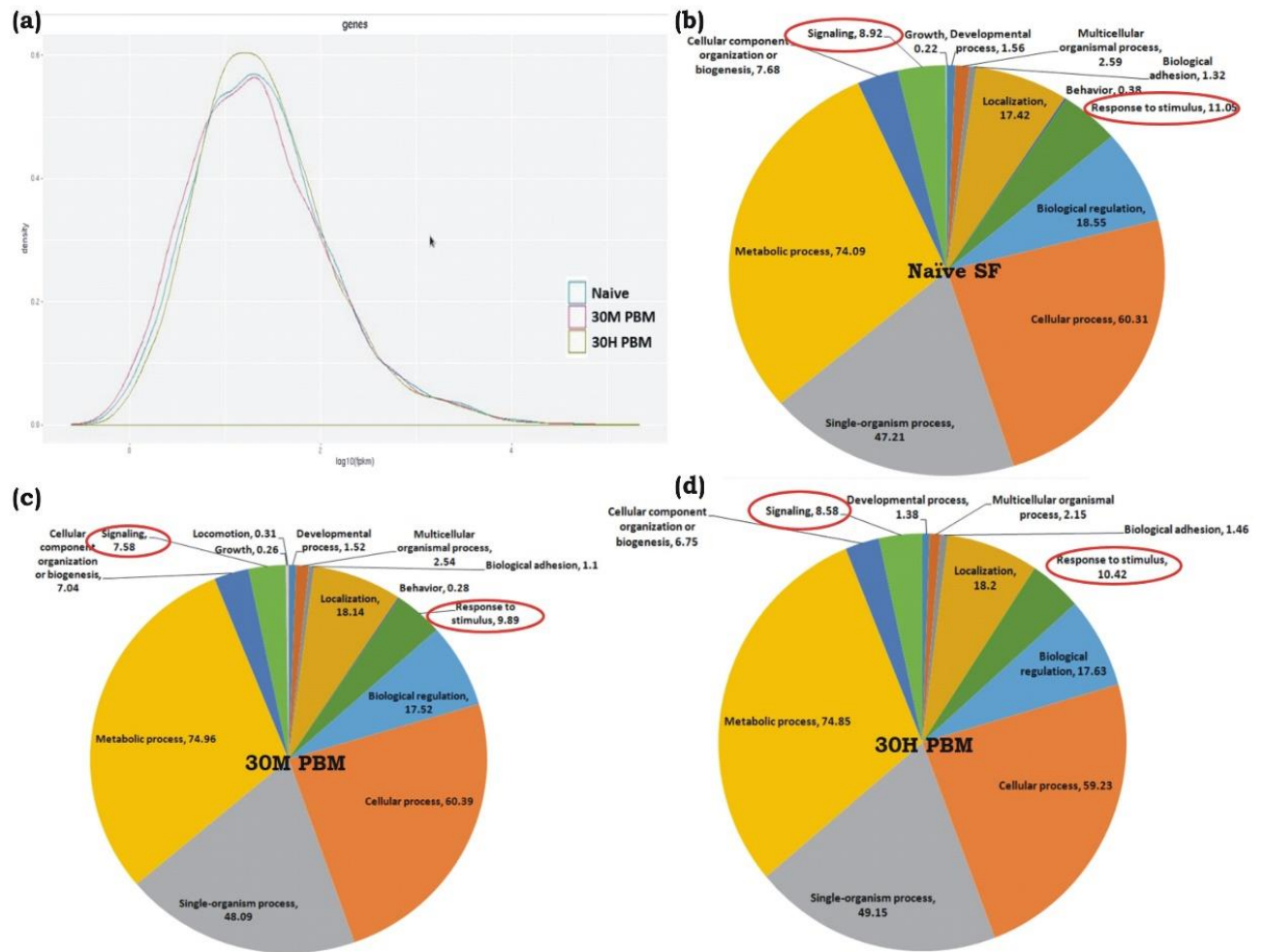


Fig. 4

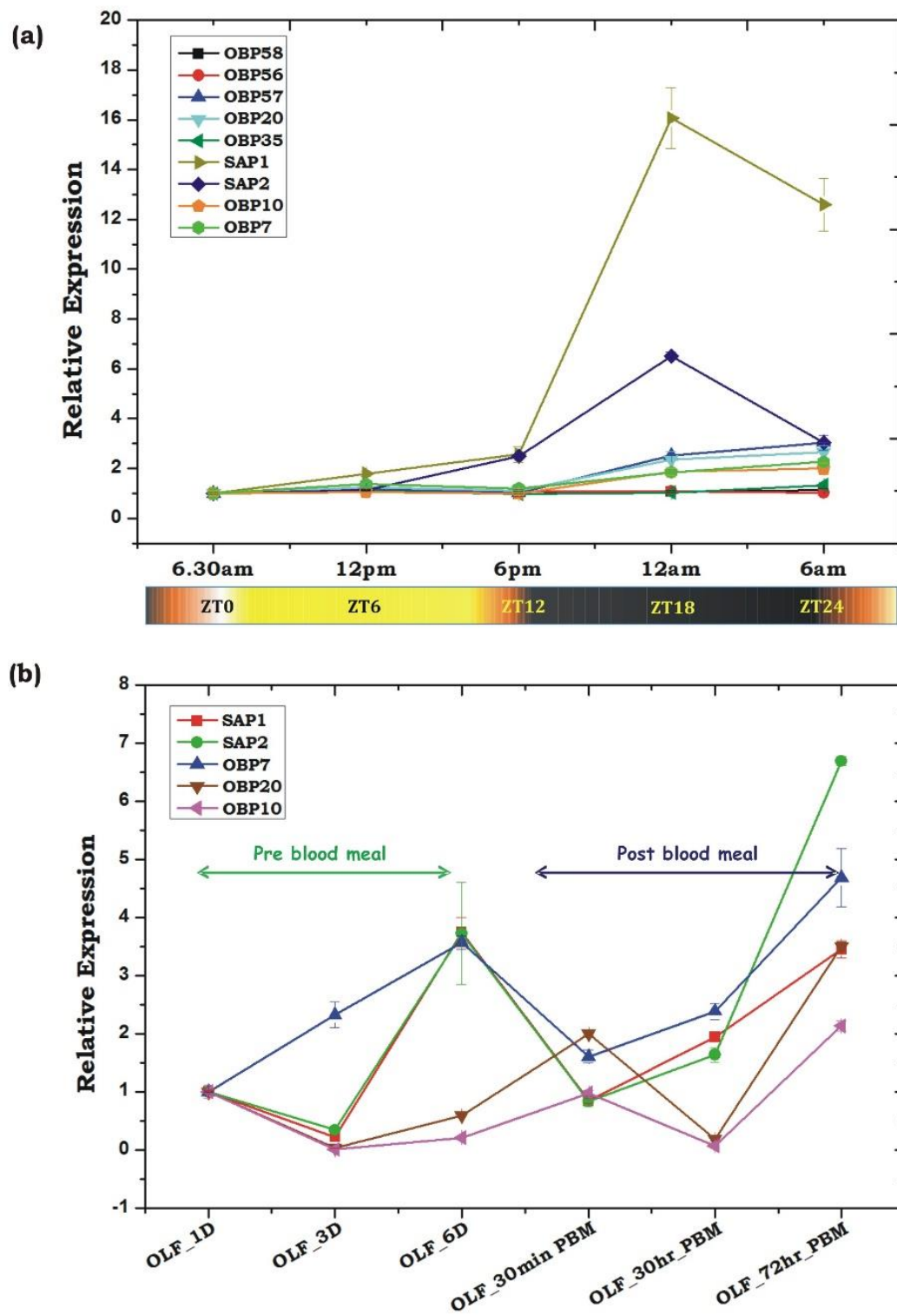


Fig. 5

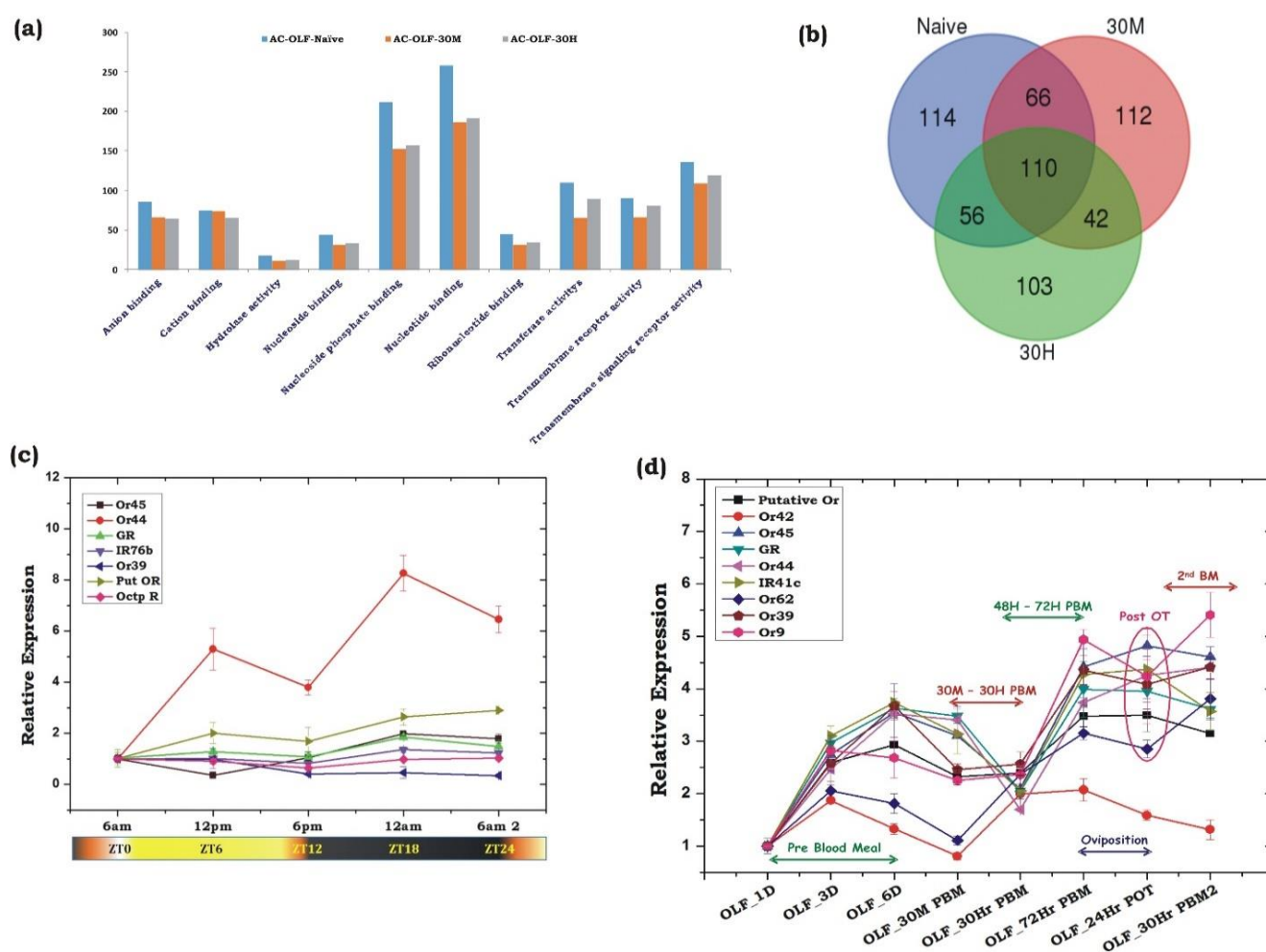


Fig. 6

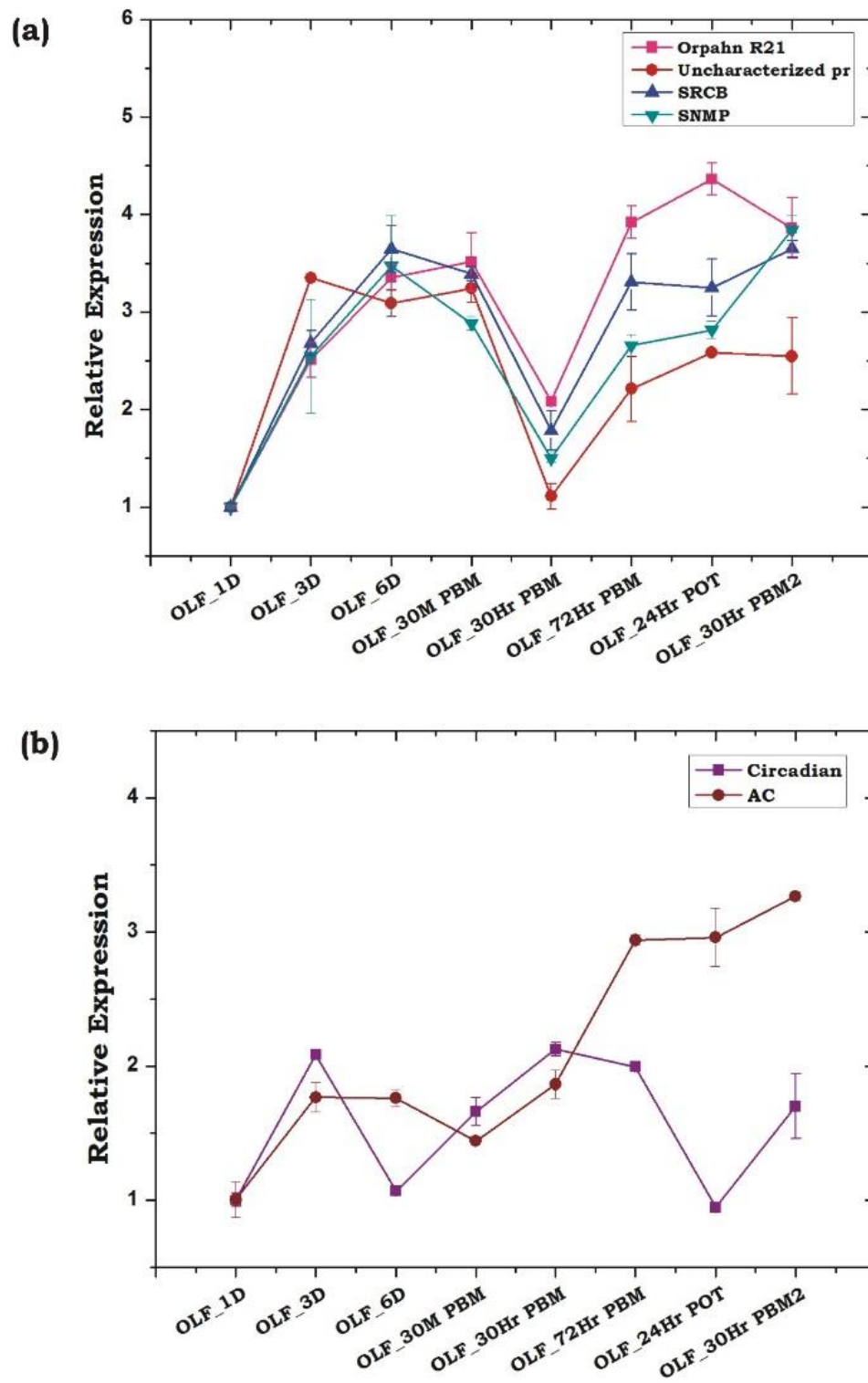


Fig. 7

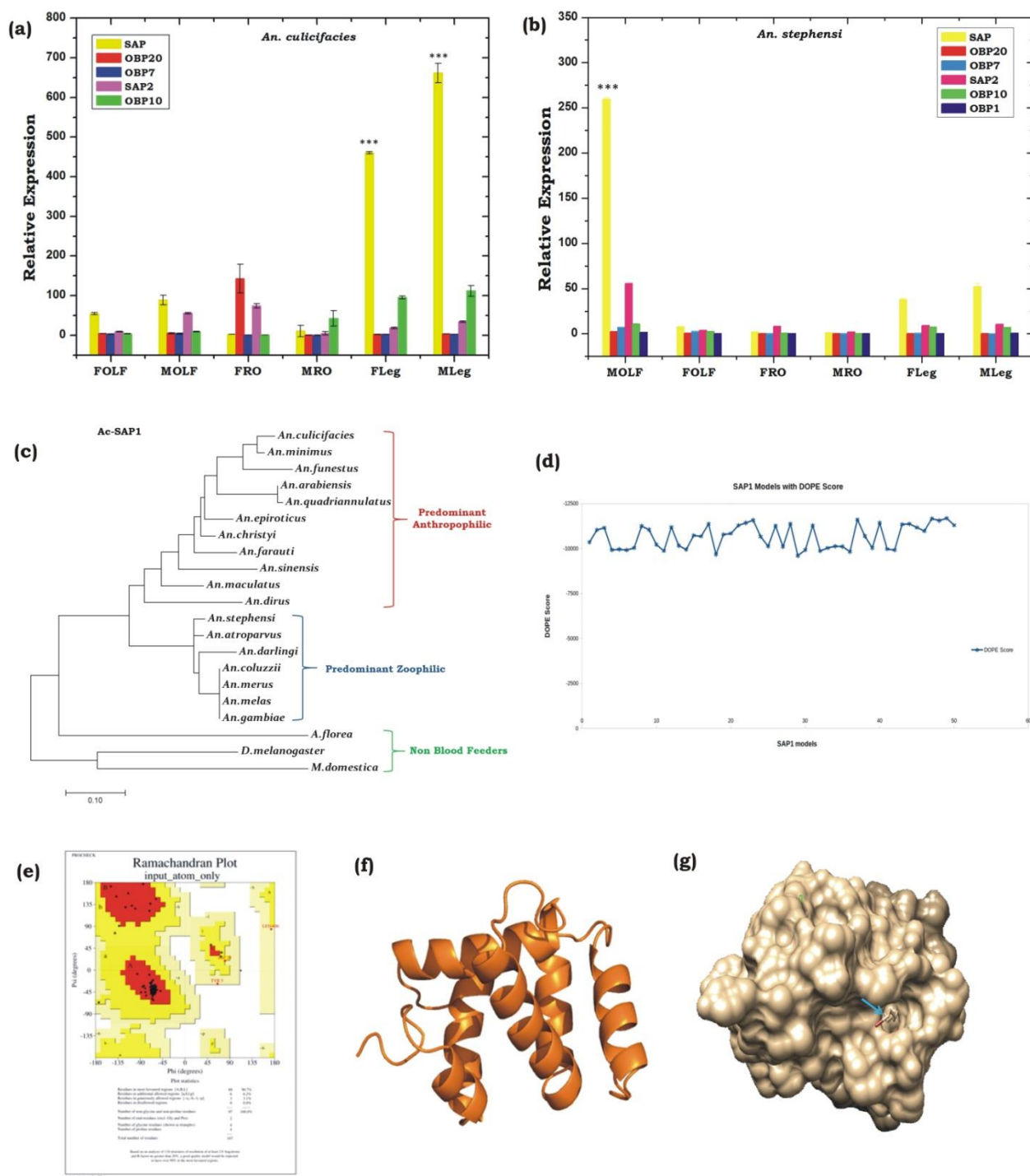
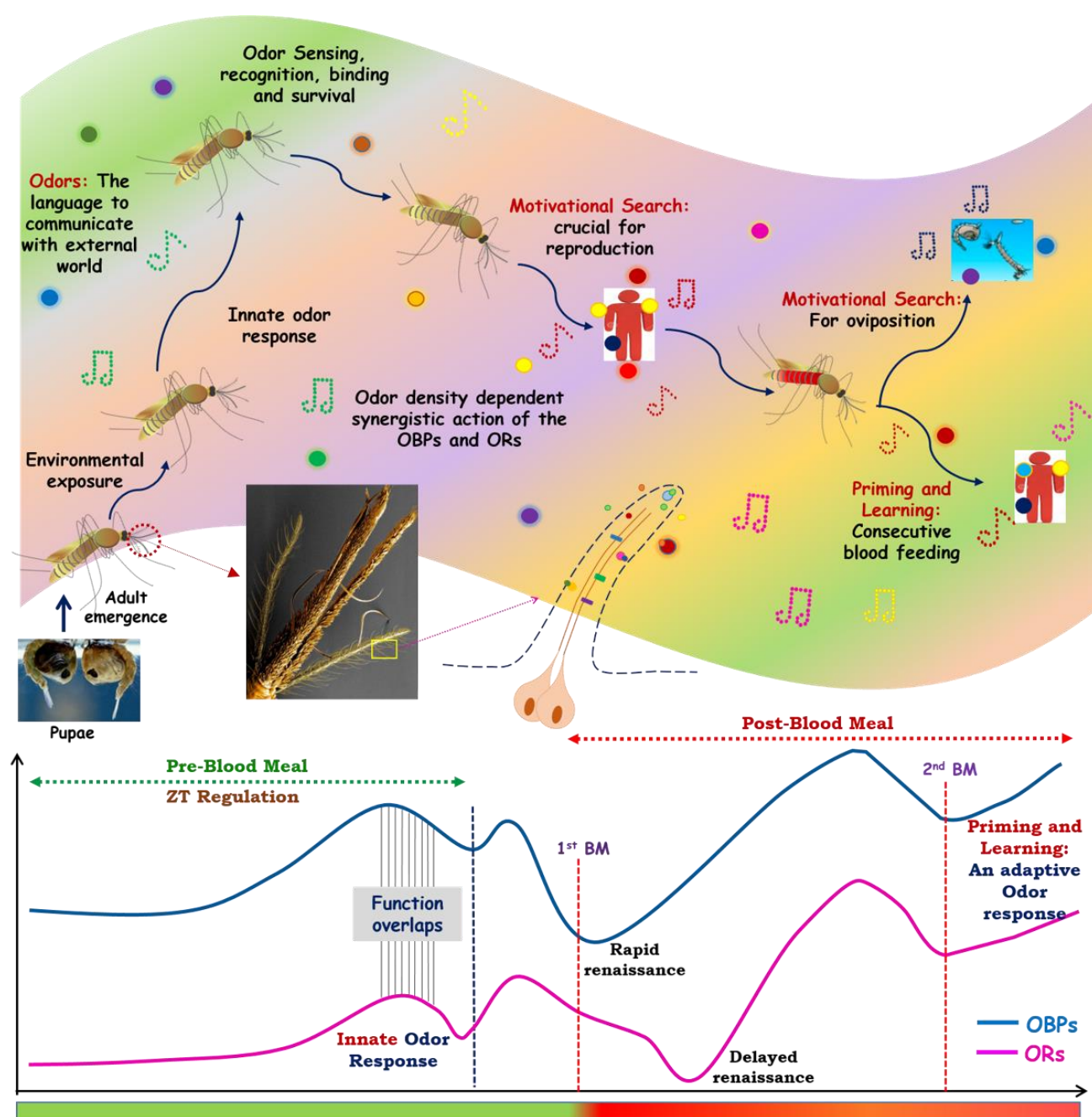


Figure 8:



Supporting Information:

A synergistic transcriptional regulation of olfactory genes derives complex behavioural responses in the mosquito *Anopheles culicifacies*

Tanwee Das De ^{1,2}, Tina Thomas¹, Sonia Verma, Deepak Singla, Vartika Srivastava¹, Punita Sharma¹, Charu Rawal¹, Seena Kumari¹, Sanjay Tavetiya¹, Jyoti Rani¹, Yasha Hasija ², Kailash C Pandey^{1,3} and Rajnikant Dixit ^{1*}.

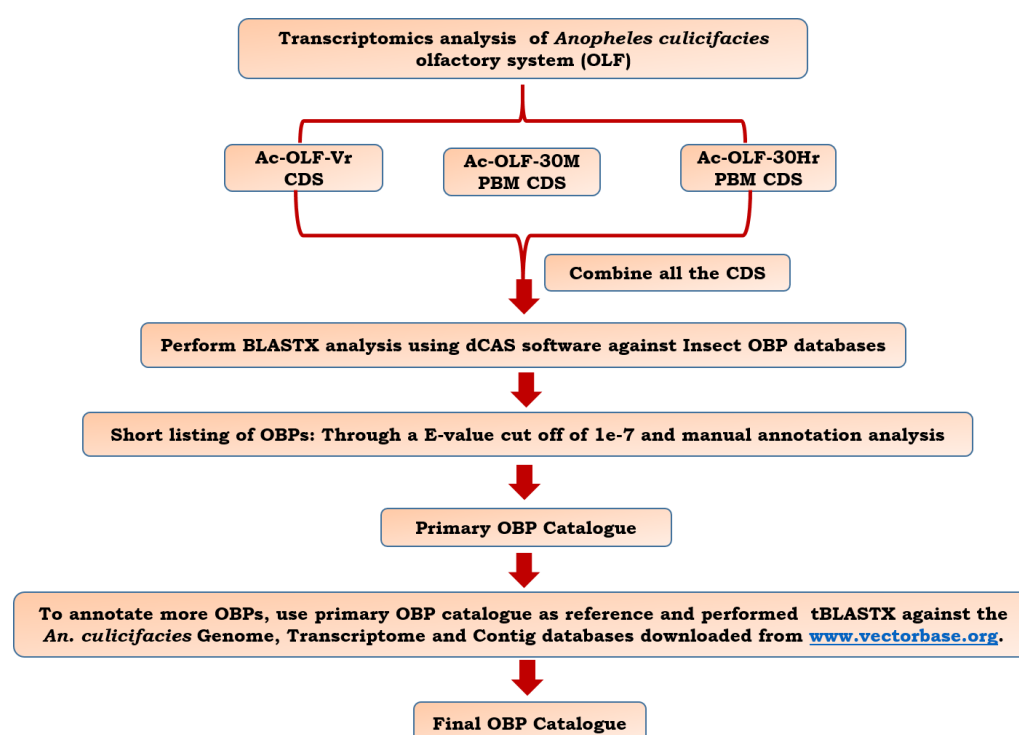


Fig. 1: Bioinformatic workflow followed to identify the odorant binding protein (OBPs) genes of *Anopheles culicifacies* mosquito. Putative OBP transcripts were identified from olfactory tissue transcriptome database by performing BLASTX analysis against the insect OBP database using dCAS software package with an E-value cutoff $1e^{-7}$. To identify and annotate more putative OBP genes from genome of *An. culicifacies*, we performed tBLASTX analysis of all shortlisted OBPs as a query against genome database, downloaded from www.vectorbase.org. Removal of all kinds of repetition was done and prepare the final OBP catalogue.

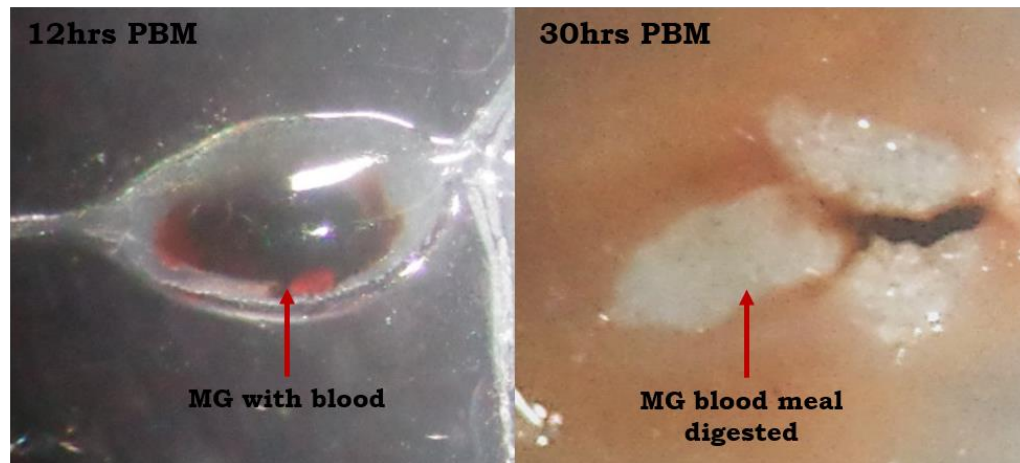


Fig. S2: Time dependent depiction of blood fed midgut (MG) showing blood meal digestion. MG digested from 12hrs post blood meal (PBM) showed undigested blood still present in the MG but after 30hrs of PBM blood meal digestion completed and thus no blood remnants is observable.

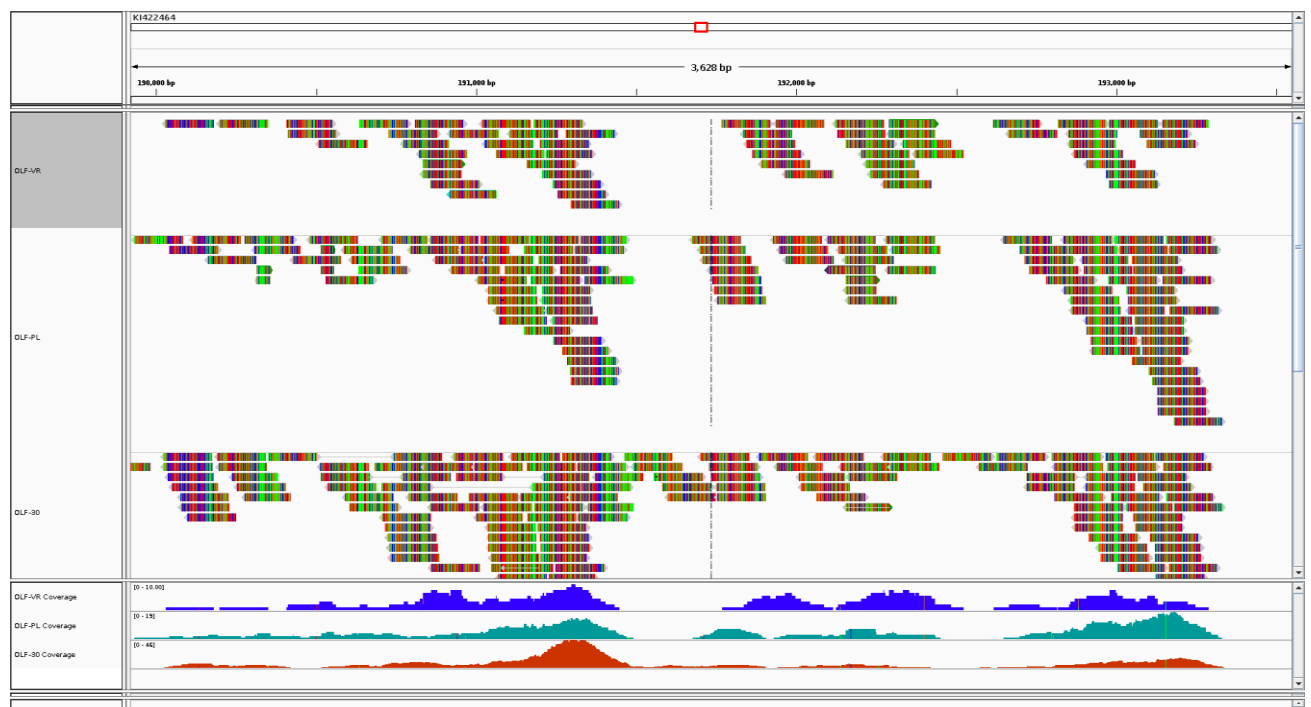


Figure S3: Self-explanatory pictorial presentation of genome guided reference mapping limitations of olfactory database of *Anopheles culicifacies*.

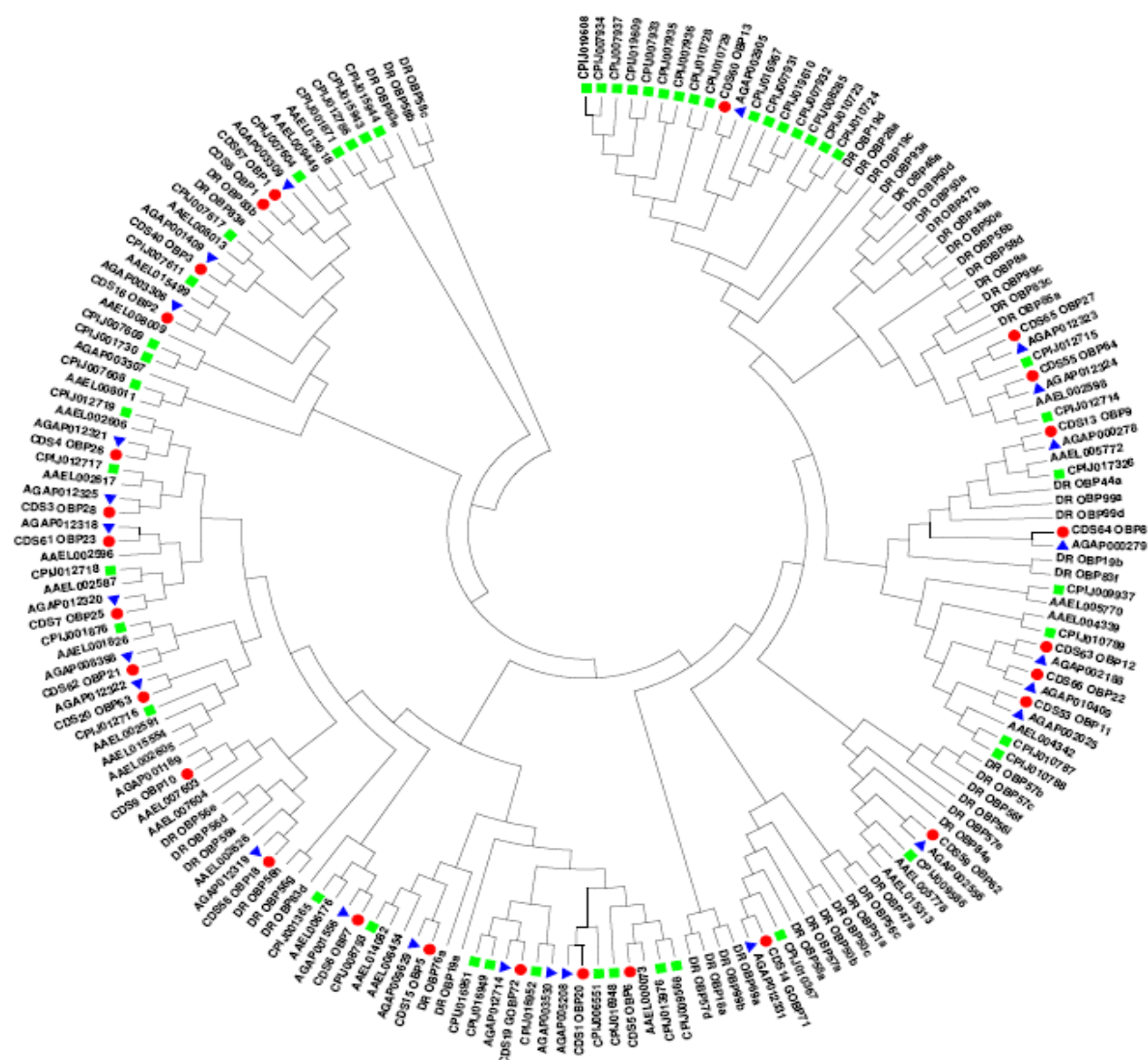


Fig. S4a: Phylogenetic analysis of Classic-OBPs. Classic-OBPs of *An. culicifacies* showed conserved sequence relationship with *An. gambiae* and other mosquito and insect species. Different color code that indicating a particular mosquito and insect species: Blue – *An. gambiae*; Red – *An. culicifacies*, Green – *Culex quinquefasciatus*. *Drosophila melanogaster* (DRX) and *Aedes aegypti* (AAX) are not marked with any color code.

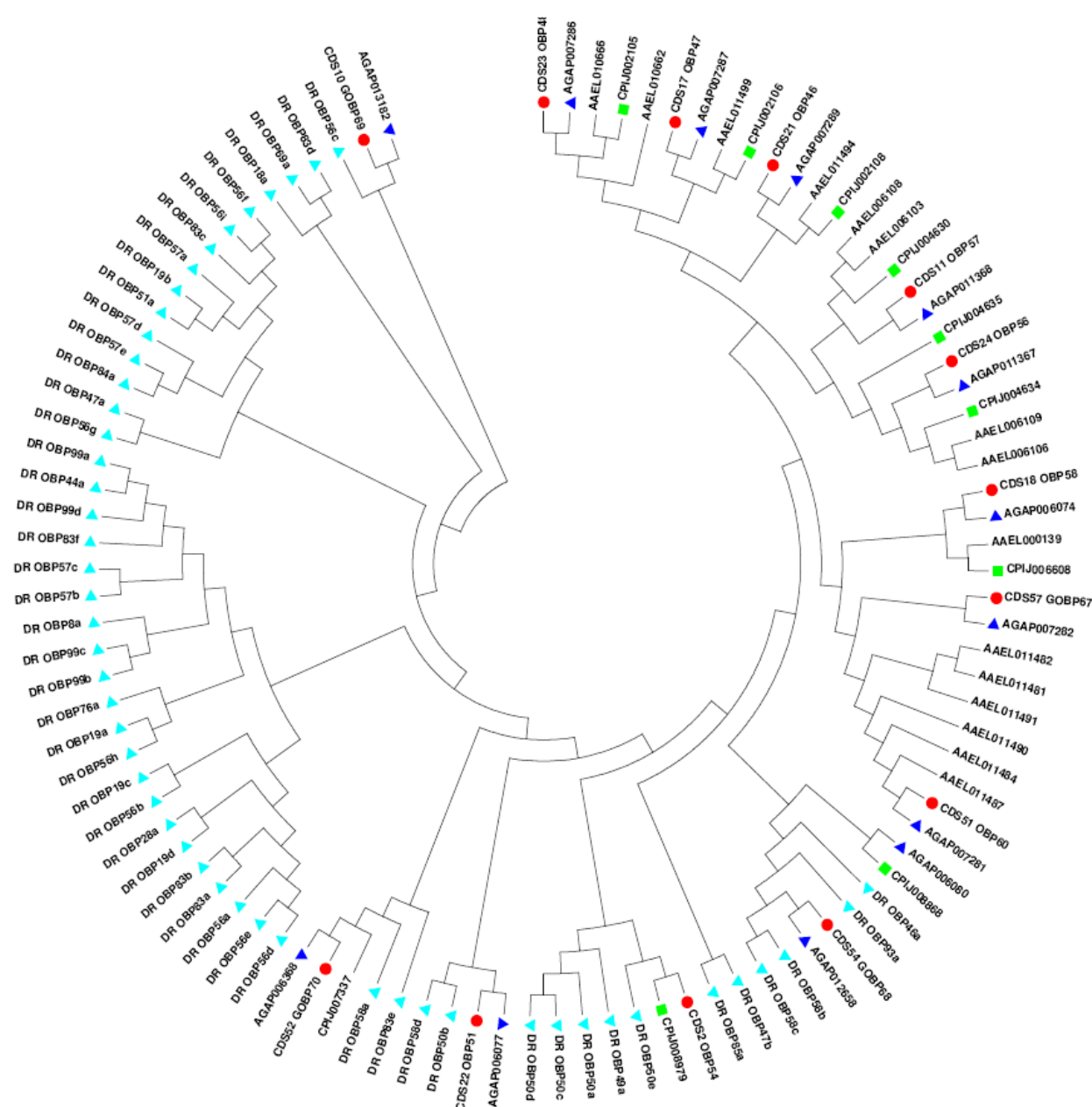


Fig. S4b: Phylogenetic analysis of Plus-C OBP superfamily. *An. culicifacies* Plus-C class of OBPs are more closely linked to mosquito specific OBPs and showed distant relationship to other non-mosquito species for example *Drosophila*. Different color code that indicating a particular mosquito and insect species: Blue – *An. gambiae*; Red – *An. culicifacies*, Green – *Culex quinquefasciatus*; Sky blue – *D. melanogaster*. *Aedes aegypti* (AAX) is not marked with any color code.

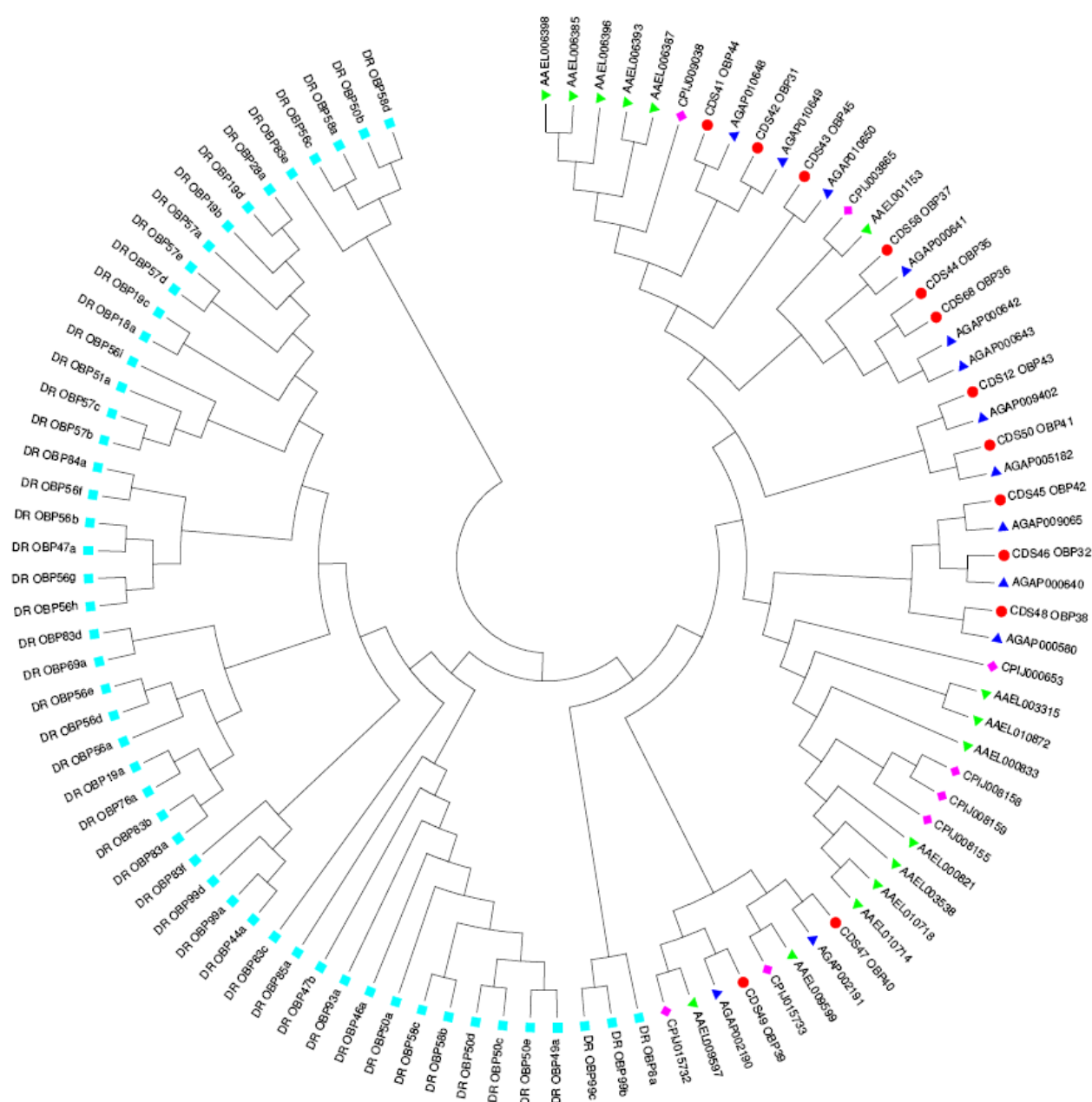


Fig. S4c: Phylogenetic analysis of Atypical OBP superfamily. *An. culicifacies* Atypical OBP class are more closely linked to different mosquito species and showed distant relationship to other non-mosquito species for example *Drosophila*. Different color code that indicating a particular mosquito and insect species: Blue – *An. gambiae*; Red – *An. culicifacies*, Pink – *Culex quinquefasciatus*; Sky blue – *D. melanogaster*; Green - *Aedes aegypti*.

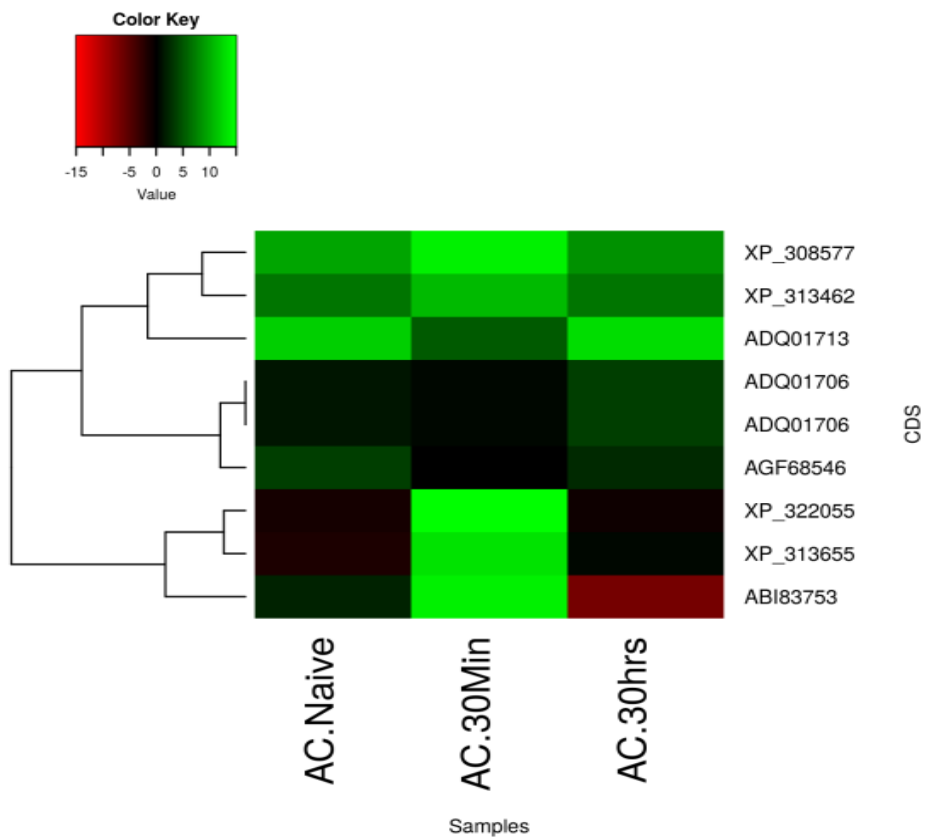


Figure S5: Transcriptional Response of *An. culicifacies* OBPs. (a) Heat map showing differential expression pattern of eight common OBP genes in naïve and blood fed olfactory tissue of *An. culicifacies*.

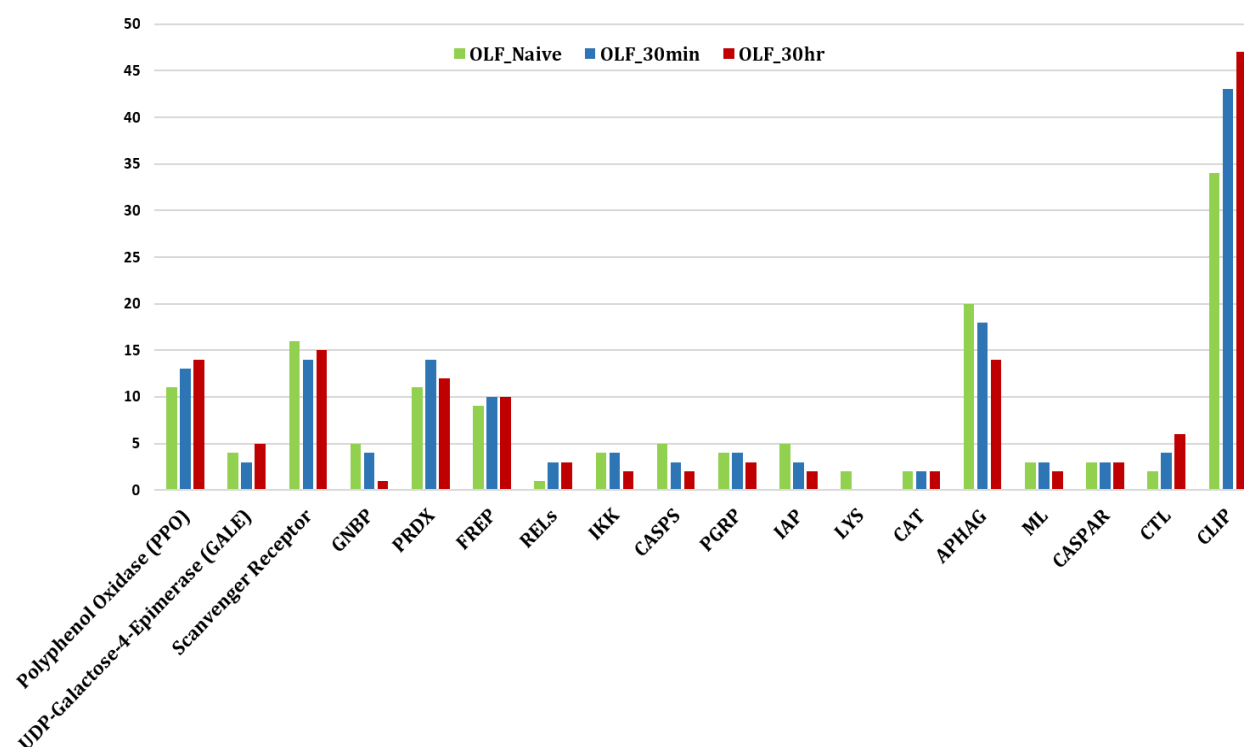


Figure S6: Differential expression pattern of olfactory specific immunome. The immune genes expressed in the olfactory system of *An. culicifacies* are retrieved through BLASTX analysis against the insect ImmunoDB database and categorized into eighteen different family members and their differential expression pattern was determined by the number of sequences appeared in each RNASeq data of naïve and blood fed mosquitoes.

Fig. S7

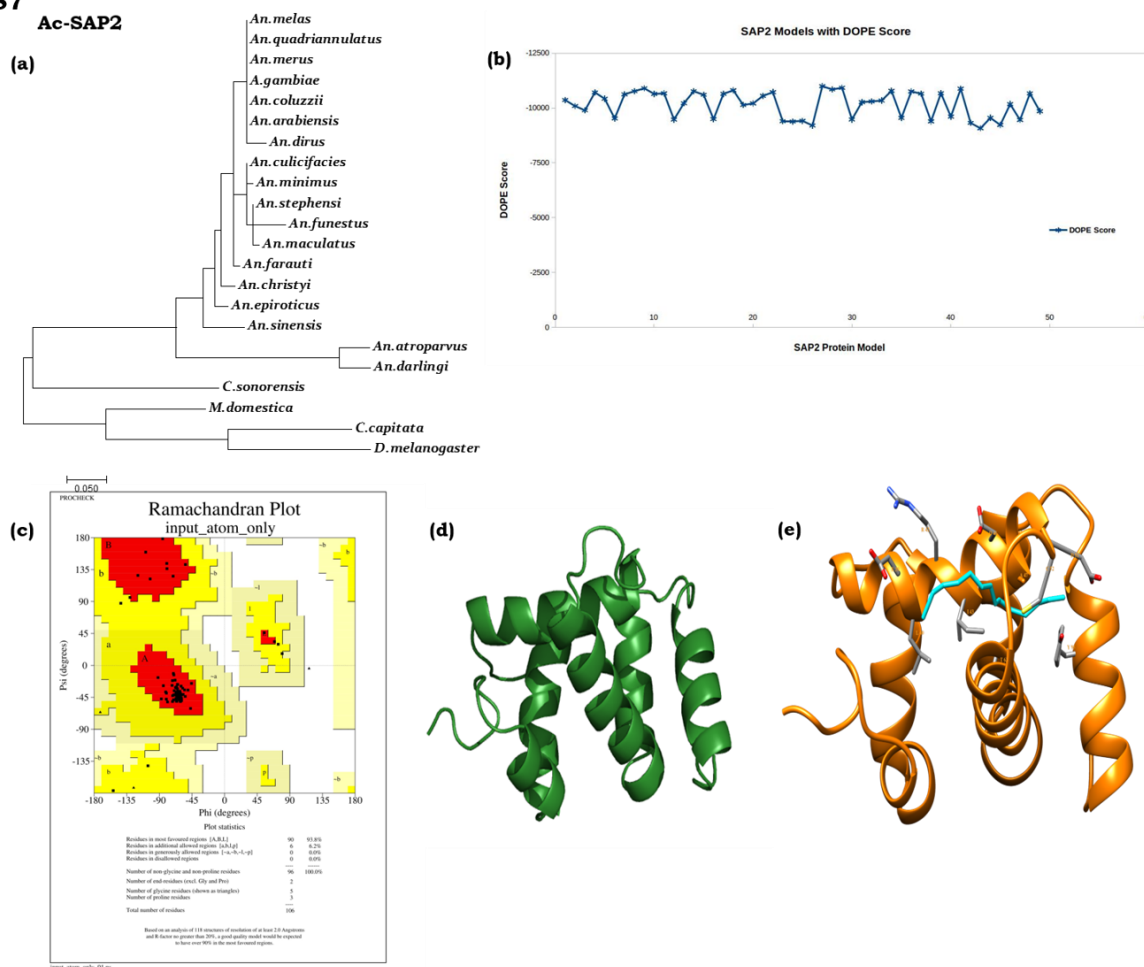


Figure S7: Phylogenetic and structural analysis of SAP2. (a) Phylogenetic analysis of *An. culicifacies* SAP2 gene (Ac-SAP2). (b) DOPE score analysis for SAP2. (c) Ramachandran Plot of SAP2 protein. (d) 3-dimensional protein structure of Ac-SAP2 protein. (e) Binding site of SAP2 protein showed in space fill with nearby residues in stick form.

Supplementary Table 1: Annotation kinetics of the RNA-Seq data

Molecular Features	Ac-OLF-Naive	Ac-OLF-30M PBM	Ac-OLF-30Hr PBM
Total Transcripts	8133	8907	8396
Total BLASTx hits (NR)	7245 (~89%)	7553 (~84.79%)	6829 (~81.33%)
Transcripts with GO Match			
Molecular Function	3946	4137	3763
Biological process	3804	3985	3566
Cellular component	2097	2201	1999
Transcript with KEGG match	2645 (~32.52%)	2975 (~33.40%)	2740 (~32.63%)

Supplementary Table 2: Percentage of Differentially Expressed Transcripts

Sample	No. of Transcripts	Transcripts showing Differential gene Expression (DGE)	Upregulated Transcripts	Downregulated Transcripts	Percentage of Transcripts showing DGE
Ac_OLF_naive vs Ac_OLF_30min	(8133 + 8907) = 17040	Total - 3749 Significant – 2540 Not significant - 1209	1042 (6%)	1498 (8.7%)	14.9% CDS show differential expression
Ac_OLF_Naive vs Ac_OLF_30hr	(8133 + 8396) = 16529	Total -3377 Significant – 2128 Not significant - 1249	396 (2%)	1732 (10%)	12.87 % show differential expression

Supplementary Table S3:

Serial No.	Transcript ID	Origin of the Transcript	Name	<i>An.culicifacies</i> Database Match	E-Value	Identity	Sub-Family	Ortholog in <i>An.gambiae</i>	FPKM
1.	Transcript _1018	30hr PBM	OBP20	ACUA000236	7E-63	95.70 %	Classic	AGAP005208	3173.27822520792
2.	Transcript _1216	30hr PBM	GOBP72	ACUA019958 *	6.00E-57	99%	Classic	AGAP012714	1254.82586
3.	Transcript _127	30hr PBM	OBP7	ACUA012847	2.00E-85	98%	Classic	AGAP001556	4144.93009
4.	Transcript _1450	30hr PBM	OBP26	ACUA003587 *	3.00E-80	98.50 %	Classic	AGAP012321	2051.81759
5.	Transcript _4885	30hr PBM	OBP63	ACUA020502 *	6.00E-70	99.30 %	Classic	AGAP012322	187.690564
6.	Transcript _6613	30hr PBM	OBP3	ACUA024106	5.00E-07	92.30 %	Classic	AGAP001409	21144.6696
7.	Transcript _91	30hr PBM	GOBP71	ACUA003567	9.00E-169	95.50 %	Classic	AGAP012331	4939.85295
8.	Transcript _109	Naive	OBP5	ACUA003763	2.00E-48	97.60 %	Classic	AGAP009629	301.156325
9.	Transcript _3113	Naive	OBP28	ACUA001691	1.00E-75	96.20 %	Classic	AGAP012325	63.5528511
10	Transcript _1290	Naive	OBP9	ACUA003296	6.00E-89	100 %	Classic	AGAP000278	389.876797
11	Transcript _138	Naive	OBP6	ACUA004501 *	3.00E-12	41.20 %	Classic	AGAP003530	238.355131
12	Transcript _270	Naive	OBP2	ACUA004189	8.00E-90	97.30 %	Classic	AGAP003306	211.717742
13	Transcript _218	30min_PBM	OBP10	ACUA017068	1.00E-71	99.20 %	Classic	AGAP001189	40.2405414
14	Transcript _48	30min_PBM	OBP25	ACUA014063	3.00E-65	99.20 %	Classic	AGAP012320	92.3071105
15	Transcript _62	30min_PBM	OBP1	ACUA014299	2.00E-87	99.30 %	Classic	AGAP003309	608.296339

16	Transcript _59	30min_ PBM	OBP54	ACUA00 1499 *	2.00E -56	97.10 %	Pluc-C	AGAP00 6080	41.4381766
17	Transcript _3357	30min_ PBM	GOBP 69	ACUA02 4897	3.00E -94	93%	Pluc-C	AGAP01 3182	323.549283
18	Transcript _207	30min_ PBM	OBP47	ACUA01 1874	2.00E -115	96.90 %	Pluc-C	AGAP00 7287	116.931
19	Transcript _7419	30min_ PBM	OBP58	ACUA01 9212 *	1.00E -109	91.60 %	Pluc-C	AGAP00 6074	28.093679
20	Transcript _5393	30min_ PBM	OBP46	ACUA02 0810	8.00E -123	95.60 %	Pluc-C	AGAP00 7289	23.4694097
21	Transcript _6620	30min_ PBM	OBP56	ACUA02 5325	1.00E -143	97.40 %	Pluc-C	AGAP01 1367	20.576198
22	Transcript _611	Naive	OBP57	ACUA02 8407	1.00E -114	97.60 %	Pluc-C	AGAP01 1368	1983.04862
23	Transcript _4488	Naive	OBP51	ACUA02 3815 *	7.00E -23	87.50 %	Pluc-C	AGAP00 6077	65432.854
24	Transcript _22	Naive	OBP48	ACUA02 5211	3.00E -59	87.30 %	Pluc-C	AGAP00 7286	1728.56394
25	Transcript _853	30hr PBM	OBP43	ACUA00 1907	0.00E +00	96.30 %	Two Domain	AGAP00 9402	90.5977525
26	Transcript _2542	30hr PBM	D7	ACUA02 5281 *	0	98.70 %	D7 Protein Family	AGAP00 6278	462.002781
27	Transcript _2682	30hr PBM	CSP	ACUA00 8309 *	1.00E -66	100 %	Chemose nsory Protein	AGAP00 1303	215.86399
28	Transcript _5645	30hr PBM	LD7	ACUA00 2223	3.00E -77	98.40 %	D7 Protein Family	AGAP00 7286	34824.3385
29	Transcript _64	30hr PBM	SD7	ACUA00 1093	1.00E -33	98.40 %	D7 Protein Family	AGAP00 8281	1348.33451
30	Transcript _1808	Naive	CSP	ACUA01 0232 *	6.00E -51	96.70 %	Chemose nsory Protein	AGAP00 8055	519.825389
31	Transcript _447	Naive	CSP3	ACUA00 4458	7.00E -71	96.70 %	Chemose nsory Protein	AGAP00 8059	195.413717
32	Transcript _702	Naive	CSP	ACUA01 7151	1.00E -29	90.90 %	Chemose nsory Protein	AGAP00 8054	203.377554

33	Transcript _12	Naive	Sensory Appendage Protein	ACUA01 8714	2.00E -40	100 %	Chemose nsory Protein	AGAP00 8051	24346.8834
34	Transcript _5005	Naive	LD7	ACUA01 8525	9.00E -100	97.70 %	D7 Protein Family	AGAP00 8279	53.6723049
35	Transcript _631	Naive	LD7	ACUA00 8578	1.00E -73	89.70 %	D7 Protein Family	AGAP02 8120	27.625451
36	Transcript _1446	30min_ PBM	CSP	ACUA01 3563 *	5.00E -80	99.40 %	Chemose nsory Protein	AGAP00 8058	149.776542

Genome Annotated OBP

Serial No.	<i>An.culicifacies</i> Transcript ID	Name	Signal Peptide	OBP Subfamily	Ortholog in <i>An.gambiae</i>
1.	ACUA027288	OBP11	Yes	Classic	AGAP002025
2.	ACUA007455 *	OBP64	Yes	Classic	AGAP012324
3.	ACUA019075	OBP18	Yes	Classic	AGAP012319
4.	ACUA007086 *	OBP62	Yes	Classic	AGAP002556
5.	ACUA016883	OBP13	Yes	Classic	AGAP002905
6.	ACUA005593	OBP23	Yes	Classic	AGAP012318
7.	ACUA011194	OBP21	Yes	Classic	AGAP008398
8.	ACUA019079	OBP12	Yes	Classic	AGAP002188
9.	ACUA022547	OBP8	No	Classic	AGAP000279
10.	ACUA009276	OBP27	Yes	Classic	AGAP012323
11.	ACUA018051	OBP22	No	Classic	AGAP010409
12.	ACUA023151 *	OBP60	No	Plus-C	AGAP007281
13.	ACUA006032	GOBP70	Yes	Plus-C	AGAP006368
14.	ACUA005261	GOBP68	Yes	Plus-C	AGAP012658

15.	ACUA013374	GOBP67	Yes	Plus-C	AGAP007282
16.	ACUA003780 *	OBP44	Yes	Two Domain	AGAP010648
17.	ACUA011054 *	OBP31	Yes	Two Domain	AGAP010649
18.	ACUA011785	OBP45	Yes	Two Domain	AGAP010650
19.	ACUA011802 *	OBP35	Yes	Two Domain	AGAP000642
20.	ACUA004895	OBP42	Yes	Two Domain	AGAP009065
21.	ACUA010086 *	OBP36	Yes	Two Domain	AGAP000643
22.	ACUA020837	OBP32	Yes	Two Domain	AGAP000640
23.	ACUA028423 *	OBP40	Yes	Two Domain	AGAP002191
24.	ACUA008136 *	OBP38	No	Two Domain	AGAP000580
25.	ACUA017746 *	OBP39	Yes	Two Domain	AGAP002190
26.	ACUA003948	OBP41	Yes	Two Domain	AGAP005182
27.	ACUA016063 *	OBP37	No	Two Domain	AGAP000641

***Newly annotated OBPs.**

Supplemental Table S4: Catalogue of Olfactory Receptor:

Sl No.	Gene Accession No.	Receptor Type	<i>An. culicifacies</i> ID	<i>An. gambiae</i> Identity	CDS Origin	FPKM
1.	XP_310061	Or29	ACUA025107	AGAP009111	Naive_Transcript_6279	26.602643
2.	XP_315068	OR35	ACUA009020	AGAP004971	Naive_Transcript_7874	11.9370834
3.	XP_315072	OR31	ACUA014252	AGAP004974	Naive_Transcript_4545	27.0352063
4.	XP_309205	OR36	ACUA003785	AGAP001012	Naive_Transcript_3706	14.6902145
5.	XP_311894	OR62	ACUA008089	AGAP011978	Naive_Transcript_4436	17.7898333
6.	XP_313640	OR26	ACUA020087	AGAP004357	Naive_Transcript_7012	19.519759
7.	XP_315773	OR33	ACUA020367	AGAP005760	Naive_Transcript_6630	19.2215628
8.	XP_317124	OR9	ACUA008607	AGAP008333	Naive_Transcript_6764	129.219612
9.	XP_321150	Gustatory Receptor	ACUA025789	AGAP001915	Naive_Transcript_6229	39.039518
10.	EFR27310	IR21a	ACUA000154	AGAP008511	Naive_Transcript_5794	8.28742772
11.	XP_311997	IR41a	ACUA014728	AGAP002904	Naive_Transcript_7850	23.907604
12.	XP_003436511	IR41c	ACUA016051	AGAP012951	Naive_Transcript_2012	1286.70859
13.	EFR22053	Ionotropic Receptor NMDAR3	ACUA007915	AGAP005527	Naive_Transcript_5355	10.5566044
14.	XP_312117	GLURIIc Ionotropic Receptor	ACUA017915	AGAP002797	Naive_Transcript_6513	32.6012782

15.	XP_312026	Putative GPCR class Orphan Receptor	ACUA015630	AGAP002886	Naive_Transcript_6435	17.9142377
16.	XP_311816	OR45	ACUA019417	AGAP003053	Naive_Transcript_2629	21.5803151
17.	XP_003436373	Odorant Receptor	ACUA019417	AGAP013396	Naive_Transcript_7336	13.7599877
18.	XP_318786	OR72	ACUA020212	AGAP009718	Naive_Transcript_4446	7.45172072
19.	AGS08024	OR41	ACUA014638	AGAP000226	Naive_Transcript_5372	39.3789123
20.	XP_310066	OR16	ACUA017005	AGAP009394	30min PBM_Transcript_4012	22.9711849
21.	XP_312289	OR 39	ACUA018263	AGAP002639	30min PBM_Transcript_1117	151.09935
22.	XP_315048	OR32	ACUA026067	AGAP004951	30min PBM_Transcript_8291	46.7776857
23.	XP_320543	Or63	ACUA028174	AGAP011989	30min PBM_Transcript_7504	13.5053686
24.	XP_321007	Or77	ACUA025905	AGAP002044	30min PBM_Transcript_3511	20.4188311
25.	XP_310173	Or2	ACUA019491	AGAP009519	30min PBM_Transcript_6920	25.7922077
26.	XP_320128	Putative GPCR class Orphan Receptor 16	ACUA001535	AGAP012427	30min PBM_Transcript_5058	9.13140271
27.	XP_308379	IR75k	ACUA022596	AGAP007498	30min PBM_Transcript_6656	9.88007955
28.	XP_003435724	IR75I	ACUA008736	AGAP005466	30min PBM_Transcript_5250	41.6081841
29.	EFR28910	Or23	ACUA005690	AGAP007797	30min PBM_Transcript_7463	19.515343
30.	XP_314478	Or44	ACUA017149	AGAP010505	30hr PBM_Transcript_8297	3270.8534

31.	XP_320541	Or61	ACUA01952 3	AGAP01199 1	30hr PBM_Transcript_3929	16.227537 7
32.	EFR26255	Putative GPCR class an orphan receptor 21	ACUA00174 9	AGAP00568 1	30hr PBM_Transcript_5866	5731.4820 1
33.	XP_309588	Putative GPCR class an orphan receptor 4	ACUA02127 9	AGAP00403 4	30hr PBM_Transcript_8157	1696.8165 9
34.	XP_320564	IR76b	ACUA01504 7	AGAP01196 8	30hr PBM_Transcript_4542	48.344539 3
35.	EFR26426	IR76a	ACUA00135 7	AGAP00492 3	30hr PBM_Transcript_7611	21.738059 8
Common Receptor Genes expressed in all the experimental conditions.						
36.	ACQ55870	Or 45	ACUA01941 7	AGAP00305 3	30hr PBM_Transcript_4029	19.020802 4
37.	XP_556129	Gustatory Receptor	ACUA02126 0	AGAP00549 5	Naive_Transcript_2080	7531.7311 8
38.	XP_320553	Or62	ACUA00808 9	AGAP01197 8	Naive_Transcript_1886	2125.3509 4
39.	ABK97614	Gr24	ACUA01049 0	AGAP00191 5	Naive_Transcript_7268	661.57491 4
40.	XP_312379	Orco	ACUA00346 9	AGAP00256 0	Naive_Transcript_972	425.79038 9
41.	XP_320874	Or11	ACUA01307 3	AGAP01163 1	Naive_Transcript_3225	341.52041 7
42.	XP_319142	Gr22	ACUA00405 4	AGAP00999 9	Naive_Transcript_1868	148.73984 9
43.	ACS83758	Or66	ACUA00031 6	AGAP00331 0	Naive_Transcript_5257	66.506607 4

44.	XP_312203	Or28	ACUA00979 6	AGAP00272 2	Naive_Transcript_2977	47.830094 4
45.	XP_314480	Or24	ACUA01191 5	AGAP01050 7	Naive_Transcript_6099	78.408311 3
46.	XP_00168872 6	Or33	ACUA02036 7	AGAP00576 0	Naive_Transcript_3900	25.280289 4
47.	XP_307763	Gr17	ACUA01976 7	AGAP00325 5	Naive_Transcript_7167	25.232859 2
48.	XP_319861	Or29	ACUA02510 7	AGAP00911 1	Naive_Transcript_5446	53.317533 4
49.	XP_321153	Or8	ACUA01438 3	AGAP00191 2	30minPBM_Transcript_17 95	259.15027 7
50.	XP_313200	Or42	ACUA02484 8	AGAP00427 8	Naive_Transcript_7008	18.125490 5

Primer Details:

Serial No.	Primer Name and Sequence
1.	OBP5_Fw: 5'CGGGAAGTAAAATGCTACAC 3' OBP5_Rev: 5'CGTAAGCTATTAACGGTGCT 3'
2.	OBP57_Fw: 5'GAAGGATTTGTCAAGCAGTG 3' OBP57_Rev: 5'GCTTACGTCCTCCTTGTTG 3'
3.	SAP_Fw: 5'CAGCAGTACACCACCAAGTA 3' SAP_Rev: 5'GAGGTAGTTGATCACCTGGA 3'
4.	SAP2_Fw: 5'AGGACAAGTACACCACCAAG 3' SAP2_Rev: 5'GGTAGTTGATCACCTTCTCG 3'
5.	OBP1_Fw: 5'ACGAAAAGCTCAAGTGCTAC 3' OBP1_Rev: 5'GGAAGTAGTGCTTTGGATCA 3'
6.	OBP56_Fw: 5'GACGGAAGAAGGAAGTCATC 3' OBP56_Rev: 5'GCAGTAACCAAAGTGAGAGG 3'
7.	OBP58_Fw: 5'CTCGTGTGAAGCAAGATGT 3' OBP58_Rev: 5'CCAAAGATGTCCTCTGCTAC 3'
8.	OBP35_Fw: 5'CTGAGTACGTCCCAAGCTAC 3' OBP35_Rev: 5'GAGTCTCAGCATTGTCCTTC 3'
9.	OBP10_Fw: 5'GGAAGTGAAGGGCTACAAG 3' OBP10_Rev: 5'ATCAGCTTGGTGAGAAACAC 3'
10.	OBP 7_Fw: 5' GTGCTTGGATGGAACCGTG 3' OBP 7_Rev: 5' GCGGGTATCACATTTATCCGG 3'
11.	OBP 20_Fw: 5' CCGTTTGCTTGGGAAAGACA 3' OBP 20_Rev: 5'GATACCGTCAGCAGCATTCC 3'
12.	Gustatory R45_Fw: 5' TGCTGGCCTCGTTAACAGTA 3' Gustatory R45_Rev: 5' CCGTAAATGCTAGCCGGAAG 3'
13.	OLF Receptor_Fw: 5' CATATGGTGTCTTATGCTCTGCT 3' OLF Receptor_Rev: 5' TGATGGGTTTCTGGGAACGT 3'
14.	Circadian PC_Fw: 5'GGGTCCTATTTGTGGTCGG3' Circadian PC_Rev: 5'TCCGTCTTGACTGGAAGCAT3'
15.	Or62_Fw: 5' TGGTATCAACGCAGAGTACA 3' Or62_Rev: 5' AGACCGAAGGTGCAGTAGTA 3'
16.	Orphan R21_Fw: 5' GATCCACGATAAGGAGTACG 3' Orphan R21_Rev: 5' TCTTCCAGAACGAGTTGAGT 3'

17.	Or44_Fw: 5' CTTCAATTTGTCGGTCTTGAT 3' Or44_Rev: 5' CCTCAAAGAACTTGCATAC 3'
18.	Adenylate cyclase_Fw: 5'TGGTATCAACGCAGAGTACA 3' Adenylate cyclase_Rev: 5' TAACCCTTCGTTTGCAGTAG 3'
19.	Or39_Fw: 5' TTCGATTCACAGAACTCCTT 3' Or39_Rev: 5' GCCTTAGCTCTTCGTTTACA 3'
20.	IR41c_Fw: 5' TACGTCATCGAGGGTATGAT 3' IR41c_Rev: 5' TAAGCCCAACGTGTCTTATC 3'
21.	Uncharacterized Protein: 5' TTTAGGTTGTCCTGCAGTC 3' Uncharacterized Protein: 5' ACTGATGTGAAGCAGAATCC 3'
22.	Or9_Fw: 5' AGATTGCCTACAACCTCACC 3' Or9_Rev: 5' CAGGATCCAGAATATGGTTG 3'
23.	Putative OR_Fw: 5' CTATCTTTGTGCATTTGCTG 3' Putative OR_Rev: 5'AGATTAAAACGCACAAGAGC 3'
24.	OR42_Fw: 5' TGGTATCAACGCAGAGTACA 3' OR42_Rev: 5' GTACAGCTGGACACTTCGAC 3'
25.	IR 76b_Fw: 5' ACATGATCTACGCGGACTAT 3' IR 76b_Rev: 5' GATCCTGCAGCTTGTACTTC 3'

Table S6 - Showing major differences between *Anopheles culicifacies* and *Anopheles stephensi*, two major Indian Malaria vectors:

<i>Anopheles culicifacies</i>	<i>Anopheles stephensi</i>
<ol style="list-style-type: none"> 1. <i>Anopheles culicifacies</i> contributes to about 60-65% of all malaria cases in India from rural to peri-urban areas and is widely distributed throughout the country. 2. Species complexity: <i>An. culicifacies</i> sp. comprised five sibling species provisionally designated as species A, B, C, D and E. 3. Host Preference: All the members of <i>An. culicifacies</i> are predominantly zoophilic except species E and rest indoor mainly in cattle sheds. Besides its low Anthropophagy, it acts as a major malaria vector due to the fact that it is found in high density. 4. Breeding preference: The preferred breeding sites are streams rice fields, seepage water borrow pits, irrigation channels, rain water collection etc. 5. Biting Behaviour: About 70–90% of <i>An. culicifacies</i> population caught during the whole night was found to feed prior to midnight during the months of January to April. Bimodal activity was seen during June and July indicating a further shift towards the second and third quarters of the night. During August–September, most biting takes place in the latter part of the night. 	<ol style="list-style-type: none"> 1. <i>Anopheles stephensi</i> is an important vector of malaria in urban areas. 2. Species complexity: <i>An. stephensi</i> Liston 1901 lack sibling species complex, but exists as two forms, the type form and the variety <i>mysorensis</i>, which are distinguished by differences in the egg length and width and by the number of ridges on the egg float. 3. Host Preferences: In cities, it is the main malaria vector and has shown an increased tendency to feed on man than on cattle unlike in rural areas. 4. Breeding preference: <i>An. stephensi</i> is found predominantly in clear water, except in some polluted, blocked cemented drains with grass growth in the non-riverine zone near Delhi 5. Biting Behaviour: Biting occurs mostly before midnight and maximum activity was observed in the first quarter of the night (1800–2100 hrs). Biting activity has been observed till third and fourth quarters of the night, though at a low rate. <i>An. stephensi</i> was active throughout the night and 39% population fed before midnight.