Molecular Analysis of Neuro-Olfactory System of Indian Malarial Vector *Anopheles culicifacies*

Thesis submitted to the Delhi Technological University

For the award of the Degree of

DOCTOR OF PHILOSOPHY

Submitted by

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April 2018

Dedicated to Maa..

DECLARATION

I, **Tanwee Das De**, certify that the work embodied in this Ph.D. thesis is my own bonafide work carried out under the joint supervision of **Dr. Yasha Hasija,** Assistant Professor, Department of Biotechnology, Delhi Technological University (DTU) and **Dr. Rajnikant Dixit,** Scientist D, ICMR-National Institute of Malaria Research (ICMR-NIMR), New Delhi for a period of July 2014 to April 2018. The complete research work was carried out at ICMR-NIMR. The matter embodied in this Ph.D. thesis has not been submitted for the award of any other degree/diploma.

I declare that I have devotedly acknowledged, given credit and refereed to the research workers wherever their work has been cited in the text and the body of thesis. I further certify that I have not wilfully lifted up some other's work, para, text, data, results etc. reported in the journal, books, reports, dissertations, thesis, etc., or available at websites and included them in Ph.D. thesis and cited as my own work.

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CERTIFICATE

This is to certify that the Ph.D. thesis entitled **"Molecular analysis of neuro-olfactory system of Indian malarial vector *Anopheles culicifacies*"** is original and has been carried out by **Ms. Tanwee Das De** under our supervision at Department of Biotechnology, Delhi Technological University, Delhi and ICMR-National Institute of Malaria Research, New Delhi for the award of the degree of Doctor of Philosophy. It is further certified that the work embodied in this thesis has neither partially nor fully submitted to any other university or institution for the award of any degree or diploma.

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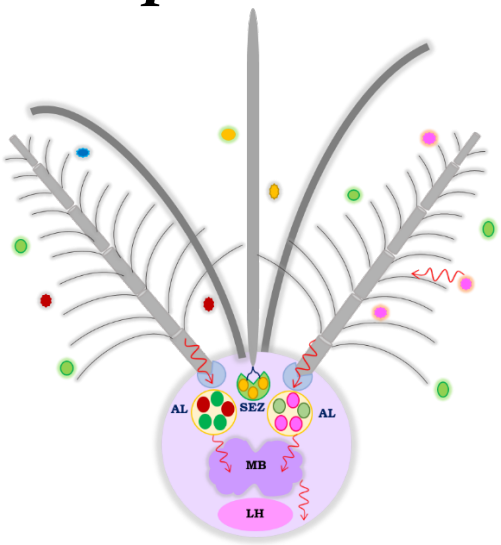

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Abstract

Mosquitoes are the deadliest animals in the world and responsible for transmitting a variety of infectious disease such as malaria, dengue fever, chikungunya, zika fever. Among them, malaria which is transmitted by *Anopheles* mosquitoes is one of the major vector-borne diseases that cause millions of mortality and morbidity worldwide. Continuous climate change, global warming, and other environmental factors are the facilitators of mosquito population growth and thus worse the situation of mosquito-borne diseases. Current tools to control and manage malaria face challenges due to the emergence of parasite resistance to antimalarial drugs and insecticide resistance of mosquitoes. Thus, alternative approaches are needed for the global elimination of malaria.

Evolution and adaptation of blood feeding behavior of adult female mosquitoes not only favored their reproductive success but also make them an important disease vectors. Mosquitoes rely extensively on their sense of smell (olfaction) for the majority of their lifecycle stages and the well-developed nasal system plays an essential role in the facilitation of olfactory guided behavior. Thus, decoding the genetic relationship of the neuro-olfactory system managing host seeking and blood feeding behavioral responses of adult female mosquitoes, may provide an opportunity to design new molecular strategy to disrupt human-mosquito interactions.

Our RNA-Seq analysis of the neuro-olfactory system of *Anopheles culicifacies* mosquito, which transmit more than 65% malaria cases in rural India, unravelled that a tight coordination of the olfactory and the central nervous system is necessary to regulate the 'pre and post' blood meal associated with complex behavioral responses such as host-seeking, blood feeding, and oviposition. A comprehensive molecular cataloging and comparative gene expression analysis of the olfactory tissue transcriptome data indicated that synergetic actions of the olfactory encoded molecular factors (Odorants Binding Proteins and Olfactory receptors) facilitate and manage the complex host-seeking behavioral events. Next, transcriptional profiling of the selected olfactory transcripts in two consecutive blood feeding experiment highlighted that adult female mosquitoes might learn and memorize from the priming effect of the first blood meal exposure, which further facilitates host selection and rapid blood meal uptake during second blood feeding event.

Furthermore, species-specific transcriptional profiling and an *in-silico* analysis of novel 'sensory appendages proteins' revealed their potential role in host-seeking and blood feeding behavior, possibly a unique target for functional characterization and designing of molecular strategy for the control of *An. culicifacies* mosquitoes. Our comparative

RNA-Seq analysis of naïve and blood fed adult female mosquitoes brain unraveled that a gradual modulation of brain transcripts expression is crucial to regulate the complex events linked to metabolic switch activities such as blood meal digestion, egg maturation, oviposition etc. Finally, the characterization of two olfactory-specific proteins *Quick-to-Court* and *Attractin* provide a new knowledge that how mosquitoes manage the conflicting demand of mating vs. blood feeding.

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ABBREVIATIONS

AC	Adenylyl Cyclase
AL	Antennal Lobe
AMS	Anopheles Male Specific
BF	Blood fed
BM	Blood meal
PBM	Post blood meal
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
Br	Brain Tissue
cDNA	Complementary Deoxyribonucleic Acid
CO_2	Carbondioxide
cAMP	Cyclic adenosine monophosphate
cGMP PK	Cyclic guanosine monophosphate Protein Kinase
CSP	Chemosensory Protein
CA	Corpora Allata
dCAS	Desktop cDNA Annotation System
DEPC	Diethyl Pyrocarbonate
DGE	Digital Gene Expression
DNA	Deoxyribonucleic Acid
Ds	Double stranded
dNTP	deoxy-nucleotide-tri phosphate
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
FRO	Female Reproductive Organ
5HTR	5-Hydroxy Tryptamine Receptor
GO	Gene Ontology
Gr	Gustatory Receptors
GABA	Gamma-Aminobutyric Acid
HC	Haemocyte
Ir	Ionotropic Receptors
IP3	Inositol Triphosphate
ILP	Insulin-like-Peptide
LN	Local Interneurons
LH	Lateral Horn
LD	Light Dark
MEGA	Molecular Evolutionary Genetics Analysis
MB	Mushroom Body
MG	Mid gut
MRO	Male Reproductive Organ
MTS	Mosquito Testes Specific
NR	Non Redundant

NCBI	National Center for Biotechnology Information
OBP	Odorant Binding Protein
OLF	Olfactory System
Or	Olfactory Receptor
ORN	Olfactory Receptor Neuron
ORF	Open Reading Frame
Orco	Olfactory Receptor Co-receptor
PCR	Polymerase Chain Reaction
PN	Projection Neuron
PDB	Protein Data Bank
RH	Relative humidity
RNA	Ribonucleic Acid
RNASeq	RNA Sequencing
RT-PCR	Reverse Transcription PCR
SF	Sugar fed
SG	Salivary Gland
Ss	Single Stranded
sNPF	Short Neuropeptide F
SAP	Sensory Appendages Protein
SRA	Sequence Read Archive
STPK	Serine Threonine Protein Kinase
TAE	Tris Acetate EDTA
TOR	Target of Rapamycin
UTR	Untranslated Region
VG	Vitellogenic Phase
WHO	World Health Organization
ZT	Zeitgeber Time

List of Publications

1. **Tanwee Das De**, Tina Thomas, Sonia Verma, Deepak Singla, Charu Rawal, Vartika Srivastava, Punita Sharma, Seena Kumari, Sanjay Tevatiya, Jyoti Rani, Yasha Hasija, Kailash C Pandey and Rajnikant Dixit. A synergistic transcriptional regulation of olfactory genes drives blood-feeding associated complex behavioral responses in the mosquito *Anopheles culicifacies*. *Frontiers in Physiology*, 2018; 9:577. doi: 10.3389/fphys.2018.00577

2. **Tanwee Das De**, Yasha Hasija, Rajnikant Dixit. Biogenic Amines in Shaping Mosquito Behavior: A Biomolecule having Pharmacological Significance. Journal of Chemical and Pharmaceutical Research, 2017, 9(12):88-92

3. **Tanwee Das De**, Yasha Hasija, Rajnikant Dixit. Transcriptional responses of *attractin* gene in the mosquito *Anopheles culicifacies*: A synergistic neuro-olfactory regulation. *Journal of Vector Borne Diseases*, 2017. (Accepted)

4. **Tanwee Das De**, Punita Sharma, Charu Rawal, Seena Kumari, Sanjay Tavetiya, Jyoti Yadav, Yasha Hasija, Rajnikant Dixit. Sex specific molecular responses of quick-to-court protein in Indian malarial vector *Anopheles culicifacies*: conflict of mating versus blood feeding behavior. *Heliyon* 3 (2017) e00361.

List of Conferences attended

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2. "Resolving the conflict of mating versus blood feeding: exploring role of *quick-to-court* gene in the mosquito *Anopheles culicifacies".* NextGen Genomics, Biology, Bioinformatics and Technologies (NGBT) Conference. Bhubaneswar, Odisha, India, 2^{nd} – 4^{th} October 2017.

Chapter 1: Introduction

1.1 Vector-Borne Diseases and Malaria

Insects are the most diverse animals that colonize on earth and also create the larger biomass of our planet [1]. The highly evolving intellectual intelligence and fast adaptive strategies make the insect's community more successful to overcome the nature's challenges. During the course of evolution, some insects have developed specialized anatomical and behavioral properties, benefiting their survival in diverse environmental conditions. The interactions between human and insects have initiated with the fate of urbanization, which may have both positive and negative impact. Though a large number of insects have strong social beneficial effects, but a few insects function as disease vectors and transmit many deadly infectious diseases such as malaria, yellow fever, dengue fever, zika fever, encephalitis, filariasis, sleeping sickness, chagas disease etc. According to WHO last annual report, malaria is one of the major vector-borne diseases that cause 212 million morbidity cases and more than 4 million mortalities. In India, malaria situation is more complex, where WHO states that the socio-economic burden of $1.94 billion due to malaria alone [2]. In the current scenario, malaria and other mosquito-borne diseases are not only restricted to the developing world but also expanded towards the developed world. Continuous climate change, global warming, rapid urbanization and other environmental factors are also accelerating the population growth of mosquitoes [3,4]. Thus, it is not hard to predict that the situation of different vector-borne diseases will worsen in the coming century.

Plasmodium falciparum and *Plasmodium vivax* are the causative agent of human malaria that are vectored by adult female *Anopheline* mosquito species. Mosquitoes get infected with the parasite when they acquire blood from an infected person and following 14-18 days of incubation, they are able to transmit the infectious *Plasmodium* sporozoites into a healthy individual [5]. To save human lives from the mosquitoes' infectious bites, advanced chemical insecticide(s) still play a central role. However, fast emergence of insecticide resistance and increased toxicants to the environment demand the development of new molecular tools. Thus for the vector biologists, it is challenging to understand and manipulate the complex biology of mosquitoes, which popularize themselves as the most dangerous animals on the earth (https://www.statista.com/chart/2203/the-worlds-deadliest-animals/).

1.2 Malaria transmission dynamics and control strategies

Although there are 460 *Anopheles* species exist in the world, but only 100 species are actually attracted towards the human and are responsible for transmitting deadly malaria diseases. *Plasmodium* parasites took an advantage of the biting behavior of *Anopheles* mosquitoes for successful completion of its sexual (sporogony) and asexual (schizogony) life cycle in the mosquito and vertebrate hosts, respectively [5]. Human urbanization and the advancement of the agricultural practices not only increased the close association among these three interacting partners i.e. human, parasite and mosquitoes but also positively affected the population size of the *Plasmodium* parasites [6]. Although *P. falciparum* is primarily responsible for most of the deaths in the African continent, however the dormancy and relapsing property of *P. vivax* facilitate their broader distribution, making them a most potent future threat for the society [7].

Thus, it is not hard to state that the control and elimination of malaria can only be possible by the strategic application of dual mode of control approaches, targeting the parasites as well as mosquitoes simultaneously. Currently, the new generation of anti-malarial drugs, such as chloroquine, primaquine, artemisinin combination therapy is widely used to treat *Plasmodium* infection. However, the rapid generation of drug resistance in parasites, poor handling, the poverty and the lack of consciousness of the target population are just some of the major obstacles to provide effective treatment in endemic areas [8,9]. On the other hand, vector management still heavily depends on the integrated application of environmental modification, chemical control by insecticides, and biological control agents such as anti-larval fish etc. [8]. An increased usage of the insecticides to control the vectors as well as pests is not only causing the emergence of the insecticide-resistant mosquitoes but also creating environmental hazards to the human health [8].

1.3 Roadblocks for malaria control and alternative approaches

Despite the above mentioned pre-genomic approaches still, there are some inherent challenges for successful malaria control. One of the major challenges includes the high genetic diversity among the *Anopheline* species which not only influences their biological adaptation in diverse ecologies but also affect the immune competency i.e. vectorial capacity for parasite transmission [10]. Insecticide resistance is another bottleneck of malaria control and eradication program [11]. Furthermore, the large genomic variation in the genome size and gene content of different *Anopheline* species, having the same number of chromosomes (2n=6), arises a question that how this variation affects the mosquitoes' behavioral biology and adaptation in diverse ecologies [10]. Therefore, a deeper understanding of the biological complexities of individual mosquito species and

overcoming the associated species-specific challenges is required for a wise manipulation in the current vector control program.

Thus using advanced genome editing CRISPR/CAS gene drive technologies, it could be possible to design novel strategies to fight against the vector-borne diseases [12,13]. The availability of genome sequence of mosquito vectors, *Plasmodium* parasites and humans have provided a significant insight to understand the molecular basis of interactions among these three interacting partners and accelerate the molecular target identification, characterization, and genetic manipulation for future malaria control. One such molecular strategy relies on the finding of key genetic factors regulating mosquito-associated complex responses including feeding behavior, pathogen-associated immune regulation, insecticide resistance and transmission etc. [8,14]. Figure 1.1 highlighted the different characteristic of mosquito biology which can be targeted for next-generation vector control measurement.

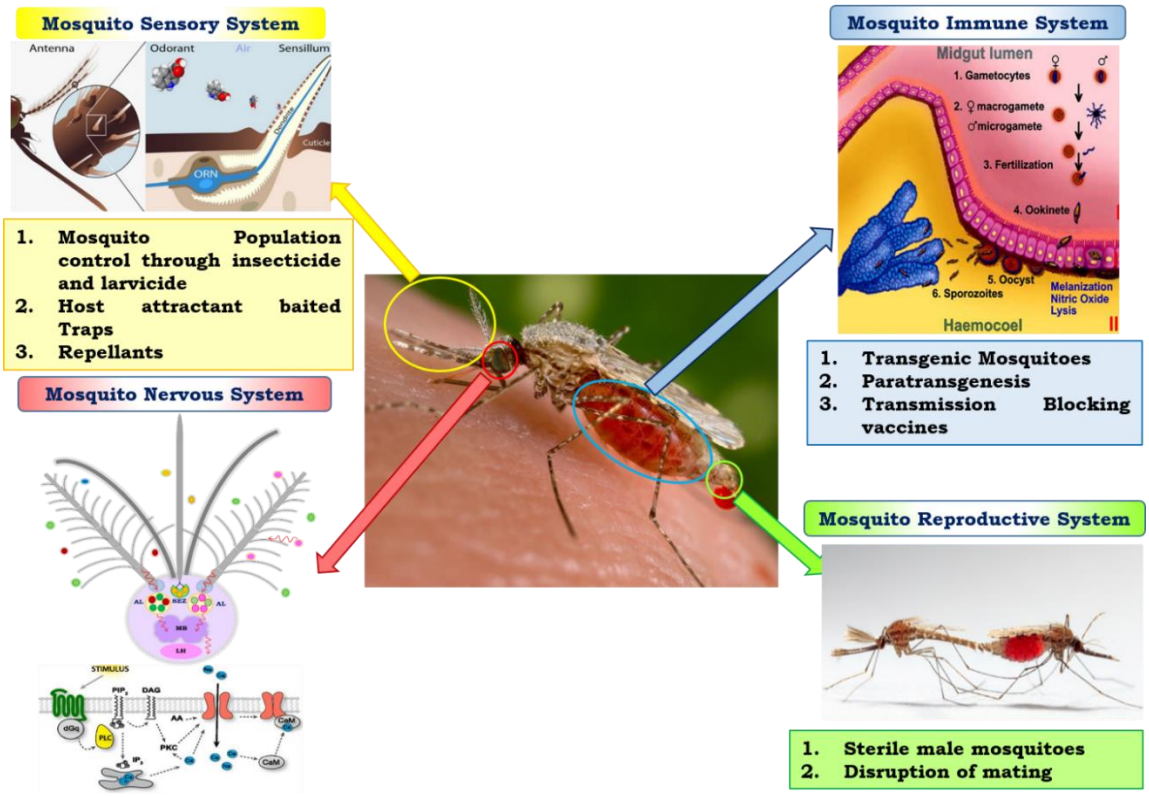

Figure 1.1: Possible mosquito control approaches in Post-genomic era.

1.4 Indian vector complexity and the biology of *Anopheles culicifacies*

Out of 58 *Anopheline* species found in India, there are six major vectors of malaria i.e. *Anopheles culicifacies*, *An. stephensi*, *An. minimus*, *An. sundaicus*, *An. fluviatilis* and *An. Dirus* [15,16]. Among them *An. culicifacies* and *An. stephensi* are the dominant malaria vectors that transmit more than 60-65% rural and ~20% urban malaria in India, respectively [17]. Importantly, both the mosquito species are getting insecticide resistant status at an alarming rate, a challenge to combat the spread of malaria. Though, both mosquito species are equally susceptible to *Plasmodium* infection but have the distinct biology of feeding, mating, breeding, vector competency, immunity & pathogen susceptibility, insecticide resistance etc. Available draft genome sequence comparison also shows that *An. stephensi* genome size (~221MB) is larger than *An. culicifacies* (~203MB), which encodes different numbers of proteins (www.vectorbase.org).

Unlike *An. stephensi* (an urban malarial vector) which is a suitable model for mosquito-parasite interaction studies as well as transgenic mosquito development, *An. culicifacies* have been poorly studied. Furthermore, *An. culicifacies* has been recognized as a complex of five sibling species provisionally designated as species A, B, C, D, and E, having distinct differences in their biological characteristics [18]. All the members of *An. culicifacies* are predominantly zoophilic except species E and rest mainly in the cattle sheds [18]. Despite of its low Anthropophagy, their presence in high density make them major malaria vector in India. *An. culicifacies* E is highly anthropophilic and is the most important and efficient vector of *P. falciparum* and *P. vivax* in southern India and Sri Lanka [18]. The bionomics of *An. culicifacies* also showed a significant difference in various regions of India which also includes the difference in their seasonal abundance, diurnal activity, human-biting behavior and vectorial capacity.

The feeding behavior of *An. culicifacies* is highly variable and greatly influenced by local ecology and climatic factors. The active biting behavior of *An. culicifacies* is in the first segment of the night in the cooler months (November-March) whereas it shifted to second to third segments of the night in the hot months (September-October) [18]. Despite these bionomic details, in-depth biology of *An. culicifacies* is largely unknown. Using advanced functional genomic approaches, recent initiatives are expected to accelerate the molecular studies focusing on mosquito–pathogen interactions, insecticide resistance and feeding behavior of Indian vectors [19–29].

1.5 Mosquitoes' blood feeding behavior and malaria parasite transmission

In nature, both adult male and female mosquitoes spend the majority of their life cycle on nectar sugar for their regular energy homeostasis. It is only the adult female mosquitoes that take a blood meal to meet the extra nutrient requirement for their egg maturation. *Plasmodium* parasites took an advantage of mosquito's blood feeding behavior to colonize and infect a new vertebrate host species. Therefore, evolution and adaptation of blood-feeding behavior in adult female mosquitoes not only facilitate mosquito population breeding but also favor malaria transmission. Thus, unraveling the tissue-specific biological complexity and targeting the key molecular factors affecting the feeding dynamics of a local mosquito vector species could be crucial to design malaria intervention strategy [30].

Mosquitoes rely extensively on the sense of smell for their every life-cycle stages including feeding, mating, and oviposition. Thus, interfering with mosquito's olfaction and disruption of vector-host interactions could be one of the key strategies for vector control. The sophisticated neuro-olfactory system of mosquitoes plays a central role during each successful feeding event by modulating and coordinating the salivary gland actions. Our previous studies on the salivary gland of *An. culcifacies* mosquito unraveled that salivary gland undergoes a gene expression switching when shifted from naïve sugar fed to blood fed status [19]. But how neuro-olfactory system regulates complex events of host seeking and blood feeding behavioral responses managing salivary glands actions in the adult female mosquitoes remains unexplored.

Immediately after emergence, an environmental exposure may favor the broadly tuned neuro-olfactory system of adult female mosquitoes to drive complex behavioral responses. The complex navigation system of the mosquito strictly depends on the coordination among multiple organs viz. host odor detection by the antennae and maxillary palp (olfactory tissue) of mosquitoes followed by decision making by the central nervous system (Brain) and lastly activate the action machinery i.e. the salivary gland for salivation, which cumulatively facilitates meal uptake. But how these neuro-olfactory derived genetic factors enable female specific 'pre and post' blood meal associated complex behavioral responses such as host-seeking, blood feeding, oviposition and re-switching to next gonotrophic cycles, are not well known (Figure 1.2).

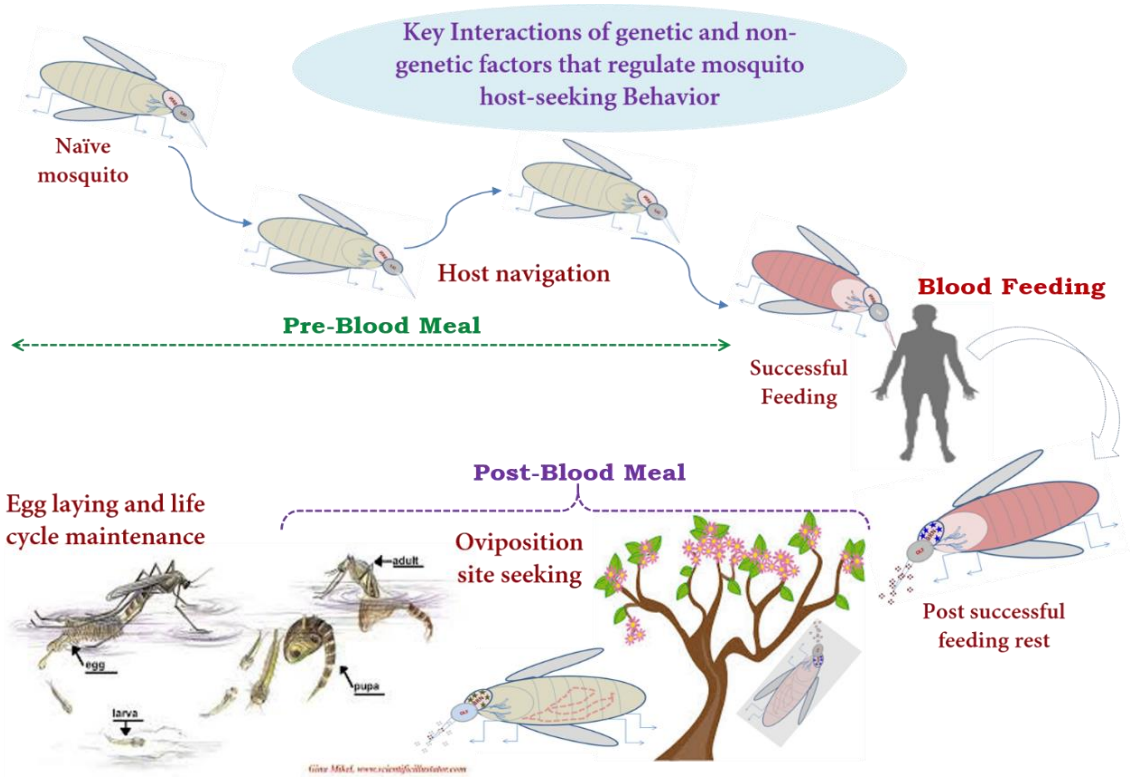

Figure 1.2: Overview of Host-seeking and blood feeding behavior associated complex events of the adult female mosquitoes

Thus, in the present investigation, we plan to decode and unravel the molecular and functional relationship of the olfactory and brain tissue regulating pre and post blood meal associated behavioral responses in the mosquito *An. culicifacies*. A comprehensive molecular cataloging and transcriptional profiling of the olfactory genes indicates that a synergetic action of the chemosensory factors (Odorants Binding Proteins and Olfactory receptors) may facilitate and manage the complex host-seeking behavioral events. Furthermore, our RNA-Seq analysis of the brain tissue suggests that blood meal causes a gradual alteration of gene expression pattern possibly to manage the physiological changes occurring in response to blood meal digestion, egg maturation, and oviposition. Finally, the characterization of two novel olfactory-specific proteins *Quick-to-Court* and *Attractin* give the glimpse of knowledge that how mosquitoes manage the conflicting demand of mating vs. blood feeding.

Chapter 2: Review of literature

2.1 Malaria and mosquito

Malaria, which is caused by the *Plasmodium* protozoa, is one of the most devastating infectious disease having a worldwide socioeconomic burden and epidemic impact. For its successful life, *Plasmodium* needs to complete both sexual (sporogony) and asexual (schizogony) life-cycles [31], occurring in the primary host (mosquito) and the secondary host (human), respectively. On the other hand, the lifecycle of all *Anopheles* mosquitoes starts with the hatching of eggs in water, which then undergoes several transition stages from larvae to pupae. The adult male and female mosquitoes are emerged through the metamorphosis of pupae and initiate their terrestrial life-cycle. Both adult male and female mosquitoes generally feed on nectar sugar sources, but only the adult female mosquitoes require a blood meal for their egg maturation. If mosquitoes take an infected blood meal containing *Plasmodium* gametocytes, the development of sporogonic cycle initiates which ends with the formation of infectious sporozoite in the mosquito host [31]. Through salivary discharge, these infectious sporozoites are injected into a healthy person during second blood feeding event, which undergoes a complex transition of asexual cycle stages causing malaria. Thus, the evolution and adaptation of blood feeding behavior of adult female mosquitoes make them the most dangerous killer in the world (https://www.gatesnotes.com/Health/Most-Lethal-Animal-Mosquito-Week).

2.2 Mosquito feeding behavior

Feeding is a very fundamental activity of every animal to achieve their optimal growth, survival and reproductive success. But the strategy of food intake and the feeding preference largely varied depending on the organism, which is also controlled by internal metabolic needs and external sensory stimulus. In the case of mosquitoes, the adult male and female sustain heavily on sugar feeding for their regular energy source for flight activity. Only adult female mosquitoes take blood meal as an optional dietary supplement for the fulfillment of their gonotrophic cycle [32–34]. Both genetic and non-genetic factors are equally responsible to shape their feeding behavior [35]. It is hypothesized that blood-feeding habit in insects has evolved when the vegetarian mosquito first bit a vertebrate host accidentally, allowing their digestive system to adapt, digest and enhance reproductive fitness through protein-rich nutrients of the blood. Another evolutionary route may be the close association of insects and vertebrate followed by adaptation and attraction towards

vertebrate cues [36]. Sugar and blood feeding of mosquitoes are two mutually exclusive events which are modulated by mosquito's internal factors such as circadian rhythm, nutritional and mating status (Figure 2.1) [33,37]. The genetic structure of mosquitoes, as well as the environmental factors such as temperature, humidity, also affect mosquito feeding behavior synergistically (Figure 2.1) [35].

Although, changes in the circadian rhythm may have a direct influence on the internal hormone level affecting sensitivity for mating and feeding in both the sexes, however it could also be important modulator to increase the host-seeking behavior of adult female mosquitoes [33]. There is very less and contrasting evidence available for defining the relationship between mating and blood feeding. Several *Culex* species are shown to change their circadian activity pattern which is mediated by the cocktail of male accessory gland protein i.e. transferred from male to female during copulation and thus activating the blood feeding behavior in females [33]. Irrespective of their mating status, the *Aedes aegypti* females are able to respond to host odor, while mating may have an additional benefit in the completion of the first gonotrophic cycle [38]. *Ae. aegypti* males are also attracted to the host odor and form swarm around the vertebrate host, predicting that mating and blood feeding may occur within the same circadian phase and at the same time [33,37].

Though the molecular factor responsible for the behavioral switch from sugar to more protein-rich blood meal has been identified/predicted from *Aedes aegypti* mosquito [39], the biology of *Anopheles* mosquito remains largely unexplored. The only circadian activity-based study demonstrated that flight activity switches from bimodal to mono-modal cycle for the successful fulfillment of blood-feeding behavior in *An. gambiae* [40–42]. Thus, the conflicting evidence from different mosquito species does not make concluding remarks about the effect of mating on host-seeking behavior.

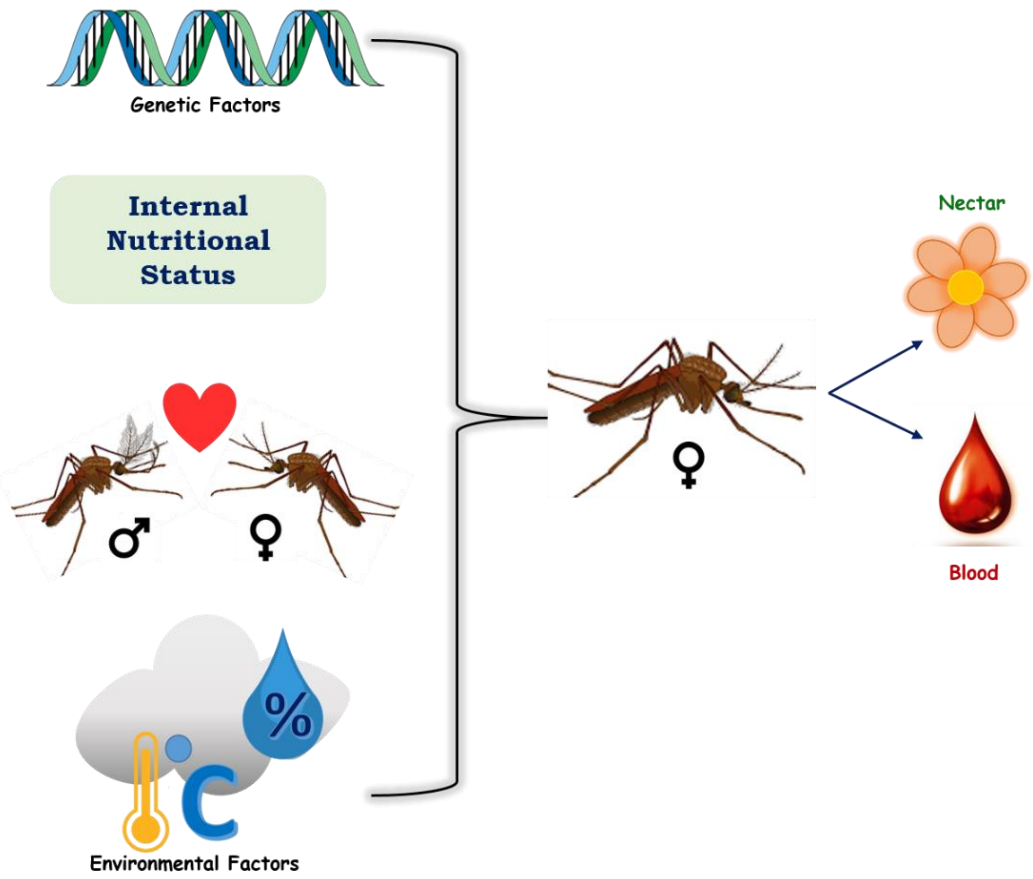

Figure 2.1: Factors affecting mosquitoes feeding behavior. The genetic structure, internal nutritional status, and the mating status cumulatively work to shape the mosquitoes feeding preference either towards sugar meal or blood meal. The external environmental factors such as temperature and humidity have an additional level of regulation which facilitates mosquito feeding.

2.3 Mosquito chemo-sensation and navigation

A well-developed olfactory system plays an essential role throughout the life cycle of mosquitoes which responds to a diverse array of biological and environmental chemical cues. These olfactory-driven factors enable mosquitoes to locate a suitable source for sugar or blood meal, to find their mate partner and the site for egg laying. Searching and locating the desired plant for sugar feeding involves both visual and chemical cues of different plant species [43]. Volatiles such as mono- and bicyclic monoterpenes are major floral odors for mosquito attraction and lighter colored plant flowers have an additional effect/benefit for successful sugar feeding [44]. But the detection of blood feeding host requires the integration of olfactory, visual, thermal and humidity cues [45,46]. A number of compounds emitted from human and other vertebrate hosts such as lactic acid, 1-octen-3-ol, carbon-di-oxide have been shown to be mosquito attractant, among them CO_2 is the most potent mosquito stimulant, that mosquito can trace from a long distance (> 10m) and

initiates the zig-zag navigation in search of the host [47]. This initial signal of CO_2 triggers the ability of the mosquito to focus on visual attraction and starting up visual flight navigation to the host until the host becomes visible (5-10m) [45]. The compound eye of mosquitoes' plays a crucial intermediate role in host localization by integrating long distance plume tracking to direct host interacting behavior that requires short-range cues viz. host skin volatiles. Heat and moisture also play a synergistic role in host localization at close host range and make the navigation successful which ends up with the action of landing and probing [45].

2.4 structure of the chemosensory system

The sensory/nasal machinery modulate the neuronal decision making events during feeding and mating which consequently affect the overall development and adaptation of mosquitoes and thus consequently, transmission of disease. [48]. Two primary components of the chemosensory system are the peripheral system where the chemical information is detected and the central processing unit where the initial signal of odor is processed. The appendages present on the head of the mosquitoes acts as the principal detection system, that includes paired antennae, paired maxillary palp and a labium [33,49]. The hair-like structures which are distributed across these appendages called the sensilla, actually housed the olfactory receptor neurons (ORNs) [33,49]. Odorants are thought to penetrate through the numerous pores present on the wall of the sensilla and then traverse through the aqueous sensillar lymph towards the array of molecular receptors present on the membrane of the dendrites of ORNs [50]. More than two decades of research on insect olfaction uncover several molecular factors that are responsible for the odor detection and downstream signal transduction processes [33,51–54]. These include Odorant Binding proteins (OBPs), Odorant Degrading Enzymes (ODEs), Odorant Receptors (ORs), sensory neuron membrane proteins (SNMPs), G-proteins, arrestins and other signaling molecules [33].

2.4.1 Odorant-binding proteins and Odorant degrading enzymes

Majority of the odorant molecules are hydrophobic in nature, thus require a cargo to traverse through the sensillar lymph to reach to the receptor moiety present on the dendritic membrane [55–57]. The role of the cargo is presumably performed by the OBPs, which are water-soluble globular proteins containing six α-helical domains with conserved cysteine residues [58]. Activation of the chemosensory receptors by odorants also require timely termination and desensitization of peripheral signaling to maintain the sensitivity of ORN-based signaling [54,59]. Odorant degrading enzymes (ODEs), particularly several esterases and cytochrome p450s, play a crucial role in this step by terminating odor-induced signal transduction processes [60–62].

In humans, only one OBP is enough to carry the odorants to their respective receptors of few hundred types. At the same time, mosquitoes and other insect's species encodes a fairly similar number (~100 – 200) of OBPs as odorant receptors [58]. Thus, the availability of this wide and diverse spectrum of OBPs in the insect's olfactory system, not only enabled their strong dependence on sensory modalities for every life cycle stages but also favored their existence in diverse ecologies. So far mosquito OBP genes are categorized under three subfamilies: (i) Classic OBPs that carry a conserved motif consisting of six cysteine residues; (ii) PlusC OBPs which contain six additional cysteines residue with novel disulphide connectivity along with three classic OBP motif; and (iii) Atypical OBPs that are the longest OBPs containing a single Classic OBP domain in its N-terminal which is extended by a C-terminal extension. Among the three subfamilies, Plus-C OBPs class is much more divergent and have only been identified from Diptera *Anopheles, Culex,* and *Drosophila,* whereas, Hymenoptera or Lepidoptera did not possess these OBPs. The first OBP from mosquito was isolated from antennae of female *Culex quinquefasciatus* (CquiOBP1) in an early twenty-first century [56,63]. The availability of genome sequence of several mosquito species in the public domain facilitates the identification and characterization of these large family of OBPs from different mosquito species (a total of 69 OBPs from *An. gambiae*, 111 OBPs from *Aed. aegypti*, and 109 OBPs from *C. quinquefasciatus*) [56].

2.4.2 Odorant receptors

Apart from the OBPs, the other principal molecule in odor detection is odorant receptors which convert the chemical signal into electrical outputs and therefore ensure the continuous flow of information from the environment to the brain [64–66]. Within the insect phylum, odorant receptors (Ors) were first identified and characterized from the model insect *Drosophila melanogaster* using intensive bioinformatics approach [67,68]. Ors are seven transmembrane domain protein with inverted topology, where N-terminus is intracellular compared to mammalian odorant receptors [69,70]. Further experimental evidence suggests that mosquito Ors act as ligand-gated ion channels, comprising heteromeric complexes of two subunits [71,72]. One subunit is highly conserved and known as olfactory receptor co-receptors (Orco), and the other subunit is largely divergent both in terms of number and amino acid sequences [64,73–75]. Pilot studies of the Or gene repertoire primarily in *Drosophila melanogaster* [68] and later in mosquitoes [75,76] and other insects [77,78] indicated that even though insects olfactory system encodes a limited number of Ors than OBPs, it is successful to respond to diverse chemical environment to meet the special demand needed at different stages of life-cycle [79]. This is only possible due to the combinatorial coding of the olfactory system of insects, enabling each Or to respond to multiple ligands, and a single ligand can activate more than one Or, resulting in a several-fold increase of odor sensitivity [51]. Thus, it is not difficult to predict that Ors

have evolved with highly sensitive and selective property for the detection of ecologically relevant odorants, which might have favored mosquito adaptation in diverse ecologies.

2.4.3 Olfactory signal transduction

The hidden information within the odor molecules is amplified by activating sensory and other neurons. The activation of a different subset of sensory neuron to a different degree forms the basis of neuronal coding. When compared with the vertebrate Ors, the insect's Ors carriers a high degree of variation with different topologies, which strongly suggest a different signal transduction mechanism [33]. Some outstanding studies highlighted that olfactory signal transduction in insects involved a ligand-gated ion channel that is formed by the heterodimerization of diverse odorant receptor and its co-receptors [59]. This fast ionotropic response does not postulate the involvement of any G-proteins and any intracellular second messengers. By contrast, another study indicated the entanglement of G-protein and the synthesis of cAMP, IP3 and other secondary messengers which together induces the downstream effector enzymes and consequently affecting the membrane potential [59,80]. The changes in the membrane potential/permeability cause the generation and propagation of action potentials along the ORN axon membrane towards the antennal lobes. In contrast to the rapid ionotropic pathway, the G protein-mediated metabotropic pathway is slower but plays an important role when the odor cues are present in lower concentration [33,59].

Apart from the Odorant receptors (Ors), gustatory receptor (Grs) [81–83] and Ionotropic receptors (Irs) [83,84] also play a critical role in the detection and response to an important class of host odors. Structurally, Grs are more related to odorant receptors and function as heteromers [85]. The detection of CO_2 also requires two functional gustatory receptor genes (Gr1 and Gr3) [48,86]. Unlike Gr and Ors, Ionotropic receptors are more ancient and evolved from ionotropic glutamate receptors. Recent studies by *Jason Pitts et.al*., in *An. gambiae* ionotropic receptors suggested that mosquitoes' Irs are responsible for the detection of amines or carboxylic acids that are involved in mediating host-seeking by adult females [84].

2.5 The brain: A neuronal decision-making unit

The discrimination and integration among the odor molecules and exchange of electrochemical information consequently influence the neuronal decision-making abilities of the brain system [87]. When an animal is given preference for food, several decisions can be made: either to eat or not, what to eat and when to eat, which not only depends on their internal physiological status but also modulated by their biological clock. In case of mosquito species, making choice among the different food source requires a fine tuning and strong integration among nasal system, internal nutritional requirement, energy expenditure and the decision making machinery.

2.5.1 Structural basis of the neuronal signal processing

The knowledge about insect olfactory coding is strongly rooted in the fruit fly *D. melanogaster*. Over the last two decades, the cellular and molecular basis of *Drosophila* olfaction have been extensively studied and comprehensively documented the three milestone of olfaction that how odor information is received, concatenated and processed by peripheral and central nervous system, respectively [88,89]. Several studies on *Drosophila* and other insects suggested that the primary brain structure which is responsible for receiving initial information of odor is the antennal lobes (ALs) [90,91]. These antennal lobes are consisting of specific numbers of spherical structures called the glomeruli. Olfactory receptor neurons (ORNs) expressing a particular type of receptor on their dendrites project their axons into the same glomerulus [33,51,78,92]. Furthermore, each glomerulus is housed with the arms of the local interneurons (LNs) and the dendrites of the projection neurons (PNs) [92]. Thus, within the antennal lobe, a synaptic connection is formed between the olfactory receptor neurons and the antennal lobe interneurons. From the antennal lobe, the olfactory information is transmitted to the higher brain center by projection neurons [33,92,93] (Figure 2.2), whereas the local interneurons facilitate inter-glomerular communication by horizontally innervating the glomerulus. The primary neurotransmitter found to communicate between local interneurons is the gamma-amino-butyric acid (GABA) and these neurons generate Na+ mediated action potential in response to olfactory stimulation [33].

The vertically arranged distinct fiber tract of the projection neurons make the connection between the ALs and the higher brain centers such as calyces of the mushroom body and the lateral horn of the protocerebrum [33]. The cell bodies of the PNs are located at the periphery of the antennal lobe glomeruli and their axons spread in the higher brain center. PNs odor responses are not identical to ORNs odor response. PNs are broadly tuned with respect to odors and respond vigorously during the odor onset [92,93]. This is due to the high convergence of ORNs expressing the same odorant receptor into a single glomerulus.

Generally, ORNs project into a particular glomerulus and PNs receives input from all of the ORN axons that enter into that cognate glomerulus and thus amplify the signal many folds which make the PNs very sensitive to small changes in the presynaptic ORNs input. Most of the ORNs to PNs synapses are cholinergic and PNs respond more strongly to the fluctuating amount of odors in the odor plume [33].

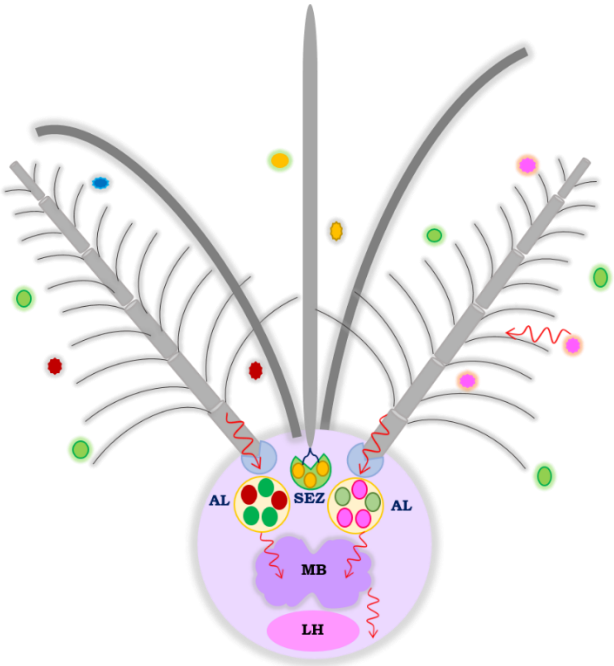

Figure 2.2: Schematic presentation of flow of odor signals from the environment to the central nervous system. Odor molecules of diverse nature (highlighted as multi-colored small circle) bind to their respective receptors present on the olfactory receptor neurons (ORNs) of antennae of mosquitoes. Then the initial signal of odor is transmitted to antennal lobe (AL) and from AL the signal is transmitted to higher brain centers i.e. mushroom body (MB) and lateral horn (LH) for signal processing and decision making. The red arrow indicates the path of signal flow.

2.5.2 Neuro-genetics of feeding behavior

Apart from the neuronal firing and neurotransmitter-mediated signal transmission which accompanies the downstream feedback for varied behavior, the molecular factors of the brain play a crucial role in neuronal decision making. The diverse neurochemicals which include neurotransmitters, neuromodulators and neuro-hormones facilitate the nervous system to transduce varied signals and thus enable the insects to manage the complex behavioral events with amazing accuracy [33,37]. Neurotransmitters are the primary and principal neurochemicals that make synaptic connections between neurons and thus maintain the continuity of the synaptic current. The crucial neurotransmitters in the insect chemosensory system are acetylcholine, gamma-amino-butyric-acid (GABA) and nitrous oxide (NO) [49,94,95].

Neuromodulators which includes the neuropeptide and biogenic amines have an intense effect on mosquito chemosensation, feeding, social behavior, circadian rhythm and also their general physiological homeostasis [37,49,96–98], [99]. Usually, these neuromodulators are produced by the specialized neurosecretory cells that are released into the local vicinity of the brain circuits and in the hemolymph. Both neuropeptides and biogenic amines modulate the response through G-protein coupled receptor-mediated signaling process [37,97]. Although, a handful of literature is available for a comprehensive understanding of neuromodulators in multiple insects [100–105] however, very limited studies have been done on blood feeding mosquitoes. In *Ades aegypti*, 28 neuropeptides have been predicted from genome database through bioinformatics approach [106], out of which neuropeptide F (NPF), short neuropeptide F(sNPF) and insulin-like-peptides (ILPs) seems to play a crucial role in mosquito feeding behavior and inhibition of host-seeking following blood feeding [107,108].

Biogenic amines are an evolutionarily conserved molecule which not only functions in neuro-signaling but also plays a crucial role in several physiological functions [109]. Most of the amines are common in both vertebrates and invertebrates (Dopamine, serotonin, and histamine), but some biogenic amines are synthesized preferentially in invertebrates (tyramine and octopamine) to perform some specialized physiological functions. Though the in-depth functions of different biogenic amines are poorly analyzed in mosquitoes, but recent studies suggested that the octopaminergic and tyraminergic signaling is crucial for oviposition and egg melanization [110]. In addition to that dopamine was found to be inversely related to host-seeking behavior [99] and serotonin play a crucial role in the regulation of salivation in mosquitoes. Neurohormones may be either local or circulatory and have a profound effect on mosquito physiological maintenance and during gonotrophic cycle [37].

Neuro-hormones are a global regulator of all life cycle processes, possibly through a controlled and precise monitoring of internal physiological status. Neuro-hormones are synthesized by specialized neurosecretory cells of the mosquito's brain. Two crucial neuro-hormones viz. ecdysteroids and juvenile hormones have a profound effect on mosquito physiological maintenance and gonotrophic cycle [37].

2.6 Food choice decision making

Food choice decision making is a complex, dynamic and random process and significantly influenced by the availability of diverse nature of food source. Integration of several internal factors is essential to modulate the food choice decision which consequently manages mosquitoes' complex behavioral properties. These internal factors include different hormonal regulating factors, neuropeptides, neuro-hormones and internal nutrient sensor molecules present in the fat body and hemocytes, the blood cells of mosquito [111–114]. Just after adult mosquito emergence, increase in the juvenile hormone (JH) synthesis from the corpora allata (CA) of the central nervous system conveys the information of the nutritional reserves, and decide the initiation of pre-vitellogenesis (PVG) process [115]. The decline of the juvenile hormone after pre-vitellogenetic phase make a clear indication about the scarcity of the nutrient content and thus ovarian resting phase (ORP) begins [115].

Although, there is no evidence exists that the decrease in the JH synthesis promotes mosquitoes towards the intake of more nutritional blood feeding, but we may hypothesize that the internal nutrient declination could promote the uptake of more proteinaceous blood meal in a demand to trigger the vitellogenic phase (VG) for the completion of the gonotrophic cycle. Neuropeptides allatostatin-C and allatostatin-A are shown to regulate JH biosynthesis inversely [116,117]. Later, *Julie L. Hentze et. al.* showed that Allatostatin-A modulates other hormonal (Adipokinetic hormone and *Drosophila* Insulin-like-peptide) activities, playing an important role by allocating the value of different nutrients (sugar and protein) to make food choice decisions [118]. The target of rapamycin (TOR) and p70S6 kinase pathway are two essential and central nutrient sensing pathway which modulate hunger and control the quality and quantity of food uptake in accordance with insulin-like-peptides [119]. Thus, it can be clearly interpreted that all of these internal factors act globally and synergistically to regulate mosquito feeding behavior and food choice decision making abilities. Apart from these internal factors, age-dependent olfactory maturation of odorant receptors, gustatory receptors, and ionotropic receptors also modulate feeding behavior in mosquitoes [120] (Figure 2.3a).

The above mentioned regulatory cascade mimics the natural scenario with abundant availability of all type of food sources, where the struggle for finding a particular food is nominal. But contrasting to this scenario, when the limited food source is available, which may bring the mosquitoes in the starvation stage. During starved conditions, neuropeptides, primarily short neuropeptide F (sNPF) and insulin-like-peptides (ILPs) and their respective receptors play a crucial role as an inherent nutritional sensor and thus modulate odor sensitivity [121–124]. But the detail model of neuropeptide mediated changing in odor sensitivity and consequent change in the host specificity are yet to be explored in

mosquitoes. Only a recent study by *Meritxell Perez-Hedo et al*. stated that starvation decreases insulin signaling and the transcript of the TOR protein which resulted in the up-regulation of Forkhead-box-binding protein (FOXO) gene in the corpora allata (CA) of mosquitoes [122]. The modulation of these genes increases the insulin sensitivity of the CA by elevating the expression of insulin receptor genes. But, this increase in insulin sensitivity either synergistically related to elevation of odor sensitivity or both are independently managed is yet to be clarified (Figure 2.3b).

The food choice decision of blood-fed mosquitoes is quite different from naïve sugar-fed mosquitoes. Blood feeding promotes the vitellogenesis process, leading to the maturation of the ovary [114,115]. Thus mosquitoes spend their more energy in finding the oviposition site for egg laying and sugar feeding is sufficient to provide the energy for the flight activity (Figure 2.3c). The previous report suggested that increase in pS6kinase and the head peptide in *Aedes aegypti* suppress the host-seeking behavior of mosquitoes as well as the biting willingness [108].

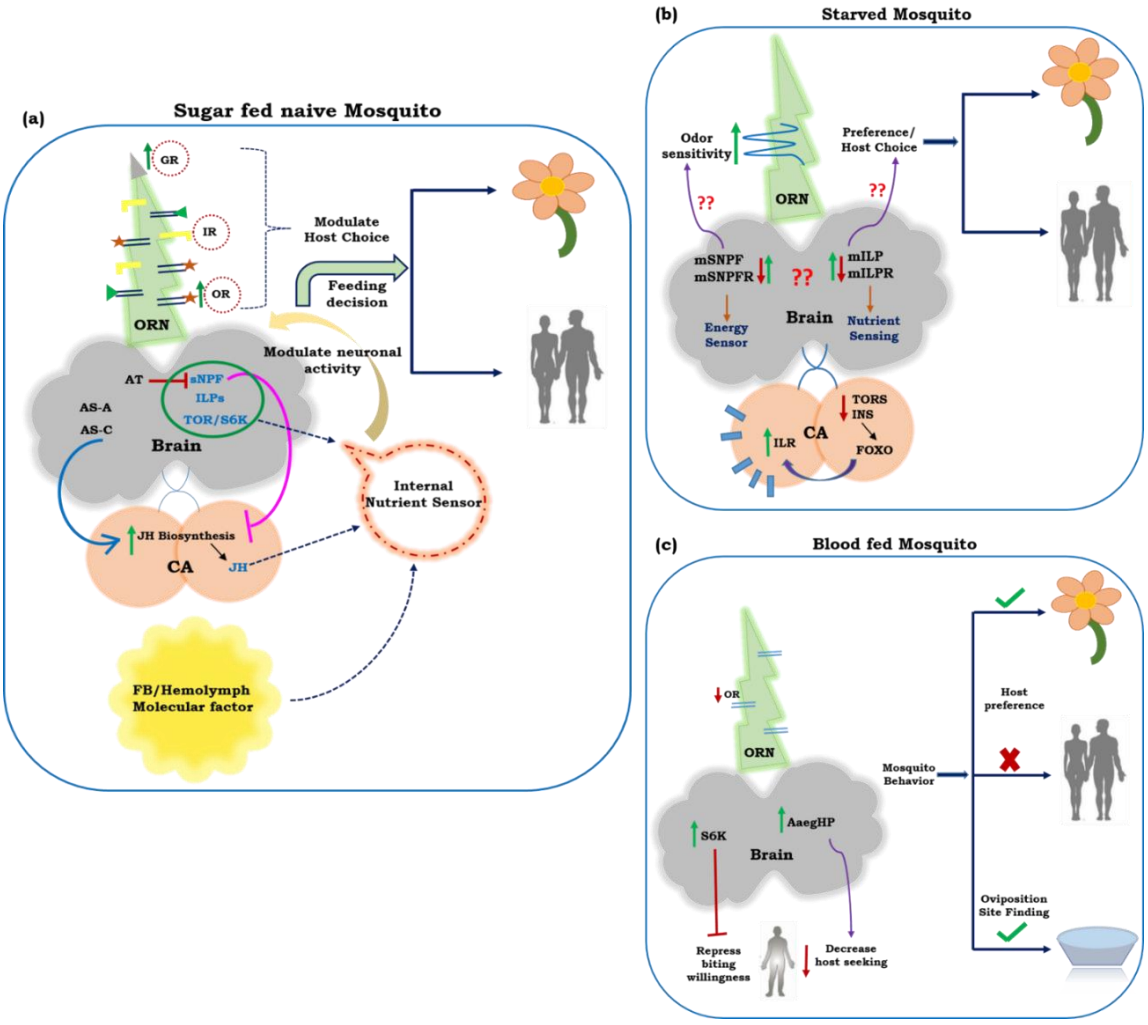

Figure 2.3: Effect of different food source on olfactory processing in mosquitoes. (a) In naïve sugar mosquitoes' juvenile hormone (JH) synthesized from the corpora allata (CA) of the central nervous system, short neuropeptide F (sNPF) and insulin-like-peptides synthesized from the brain act as some internal nutrient sensors, which can sense and regulate feeding preference. The TOR pathway and S6 kinase (S6K) also nutrient sensors which along with the hemolymph and fat body molecular factors regulate mosquitoes' feeding choices. The expression and maturation of a particular type of odorant receptors (ORs), gustatory receptors (GRs) and ionotropic receptors (IRs) also modulate mosquitoes' food specific (nectar sugar or vertebrate hosts for blood meal) choices. (b) In the case of starved mosquitoes, the elevation in the synthesis of insulin-like-peptide receptors (ILR) gene increases the sensitivity of CA to insulin-like-peptides. But these increase in sensitivity towards insulin-like-peptides would also increase the odor sensitivity of the olfactory receptor neuron or not is yet unknown. The role of sNPF, which is an energy sensor, in modulating the odor sensitivity towards either nectar or blood feeding host is also questionable. (c) The food choice decision of blood-fed mosquitoes is quite different from either sugar fed or starved mosquitoes. The increase in the S6Kinase (S6K) activity and head peptide (HP) in the brain repress the biting eagerness and thus decrease the vertebrate host-seeking behavior and activate the oviposition site finding behavior.

Chapter 3: Knowledge Gap and Working Hypothesis

3.1 Knowledge Gap

Given to the availability of knowledge and research status above, it is easy to state that the olfactory-driven factors are central in shaping every behavior of mosquitoes including feeding, mating, breeding etc. Though several international laboratories are working on insect's olfactory system, however, there are still major gaps exists in the understanding of the mosquitoes' neuro-olfactory regulation managing host seeking and blood feeding behavior, which are as follows:

1. Globally it is largely unknown that how the genomic variation affects the behavioral biology and adaptation of local *Anopheline* mosquito vector species?
2. The molecular basis of evolution and adaptation of blood-feeding behavior in the adult female mosquito is largely unknown.
3. How adult female mosquito's olfactory system manage the conflicting demand of mating vs. blood feeding is largely unexplored.
4. How system biology of mosquitoes functions to manage the coordination between the internal nutritional demand and external sensory stimuli.
5. Most of the molecular and physiological studies of mosquito olfaction are limited to African malarial vector *Anopheles gambiae* and dengue vector *Aedes agypti*. However, our knowledge on the molecular studies in the Indian malarial vectors *Anopheles stephensi* and *Anopheles culicifacies* is largely unexplored.

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3.2 Mosquito neuro-olfactory complexity & key hypothesis

Each of the feeding events of mosquitoes is initiated by random navigation, which becomes specific when triggered by a certain group of chemicals. The detection of other additional cues such as heat, moisture by the olfactory system facilitates the downstream events of host localization, landing over the host and searching for a suitable site for punching by the proboscis to initiate blood feeding. But, a successful navigation does not always corroborate with a successful feeding event, because it involves another level of regulation of the central nervous system by discriminating the odor molecules and make a decision for either to feed or not. Post landing and piercing on a particular site of the vertebrate host, successful uptake of blood meal largely depends on the proper functioning of the salivary gland which acts as the final action machinery by facilitating the feeding process through salivation.

Furthermore, post blood meal mosquitoes undergo two major behavioral switching events (i) search for the suitable site(s) for temporary resting and egg maturation (48-72hrs); and (ii) find a proper oviposition site for successful egg laying. After completion of egg laying event, the adult female mosquitoes regain their host-seeking activity for a second blood meal to complete the next gonotrophic cycles. But, how the olfactory system, brain, and the salivary gland integrate with each other to make a feeding event successful is largely unexplored.

We have recently demonstrated that adult female mosquitoes are evolved with the unique ability of salivary gland gene expression switching to manage meal specific 'prior and post' blood meal responses [19]. Here, we further extended this idea to decode and trace the possible molecular link that how the neuro-olfactory factors of adult female *An. culicifacies* mosquitoes drive sex-specific host seeking, blood feeding and oviposition behavior. To establish the plausible mechanism, we developed a working hypothesis (Figure 3.1) and planned to compare the transcriptional responses of the olfactory (detection machinery) and the brain (decision machinery) system coordinating and regulating the meal specific salivary actions in the adult female mosquitoes.

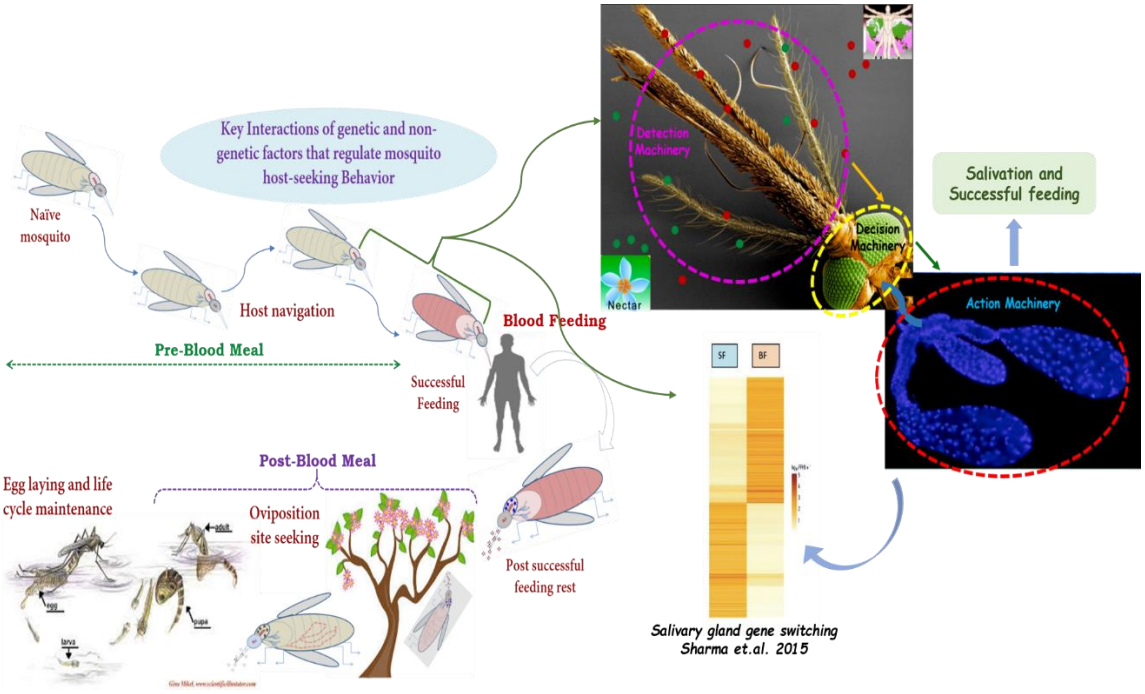

Figure 3.1: Proposed working hypothesis: Adult female mosquitoes need to manage distinct behavioral responses such as sugar feeding, host-seeking and blood feeding, oviposition site finding behavior etc. The tripartite interorgan communication and coordination among olfactory tissue, the central nervous system (brain) and the salivary gland are crucial for the completion of each prior and post blood meal associated behavioral events. Originally, the idea of decoding neuro-olfactory regulation emerges from the demonstration of salivary gene expression switching in response to prior and post blood meal [19]. The olfactory tissue (Highlighted by a purple circle) sense and bind to odor molecules emanating from either plant of vertebrate host and send the respective signal towards the brain system (Highlighted as yellow circle). After processing the initial signal of odor in the central nervous system the decision making process occurs and brain then send the signal towards salivary gland (Highlighted as red circle) and the process of salivation started to facilitate feeding. Photo credit goes to Zwiebel Lab, Vanderbilt University for the olfactory system of *Anopheles* mosquito. The salivary gland picture was taken from the research article by *Anil K. Ghosh et al*. [125].

Chapter 4: Objectives

To decode and trace the possible molecular links between olfactory perception and neuronal decision making events affecting each successful feeding event of mosquitoes, we planned the following objectives:

i) Generation of transcriptome database of olfactory and brain tissue of *Anopheles culicifacies* mosquito

ii) Comprehensive bioinformatic analysis: Molecular cataloguing and Functional annotation of the transcriptomic database.

iii) Digital Gene expression analysis and transcriptional responses of selected olfactory/brain-specific genes under different experimental conditions.

iv) Attempt for understanding the functional role the key selected genes linked to the regulation of mosquito feeding behavior.

To achieve the targeted goals, we have designed and performed a comprehensive RNA-Seq analysis of the neuro-olfactory tissues and cataloged, identified and profiled the selected transcripts expression modulating in response to blood feeding. For better presentation and understanding, a comprehensive overview of each tissue-specific biology, working hypothesis, methodology, and data interpretation has been described as an individual chapter.

Chapter 5: Decoding the Genetic Basis of Smell Detection in Indian Malarial Vector *Anopheles culicifacies*

5.1 Introduction

Evolution and adaptation of blood feeding behavior not only favored the reproductive success of adult female mosquito's but also make them potent transmitter of many deadly pathogens, killing millions of peoples globally. Decoding the molecular basis of this host seeking and blood feeding behavior 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 successfully.

Immediately after emergence from pupae, an exposure to diverse environmental/chemical cues facilitates olfactory maturation and learning of the adult mosquitoes for different innate behavioral activities such as feeding and mating in both the sexes [35]. Once, a mosquito takes first blood meal it needs to manage major physiological activities linked to blood meal digestion and egg maturation. 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 [126]. 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 [127,128].

Notably, 'prior and post' blood meal associated habitats may have a significant difference in their physical, chemical and biological characteristics [129], but how mosquito manages these complex events is still not well understood. A handful of literature in *An. gambiae* and *Aedes aegypti* suggest that mosquito peripheral sensory appendages encode molecular factors such as Odorant Binding Proteins (OBPs) and Odorant Receptors (Ors), which undergoes unique changes in response to blood meal [127,130,131]. However, it remains challenging to understand that how a sex-specific evolution of olfactory appendages including proboscis, drives complex events of pre and post blood meal associated

behavioral events in the adult female mosquitoes. 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 test and decode this sex-specific evolutionary specialty we developed a working hypothesis (Figure 5.1), a plausible mechanism which may have a significant influence on mosquito feeding and survival in diverse ecologies.

To establish a possible molecular relationship managing 'prior and post' blood meal behavioral events, we performed a RNA-Seq transcriptomic analysis of the olfactory system of adult female *An. culicifacies* mosquito, responsible for more than 65% malaria cases in India [18].

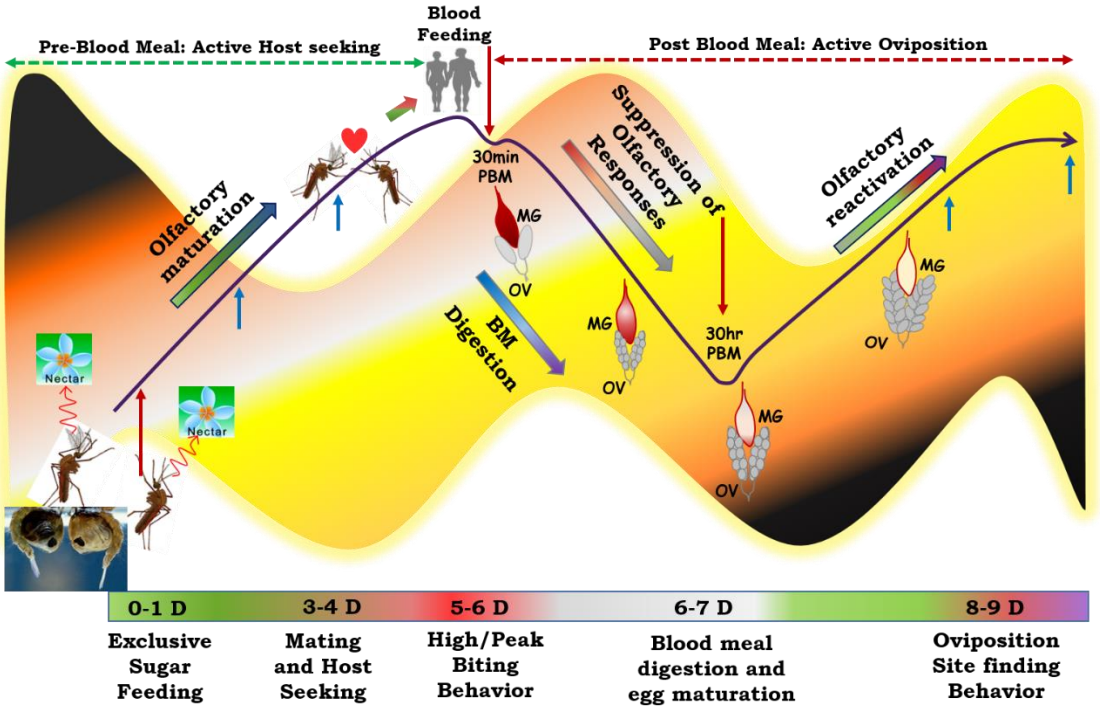

Figure 5.1: Working Hypothesis to establish functional co-relation of the olfactory system under distinct feeding status. Mosquitoes Adult mosquitoes, just after emergence from pupae are exclusive sugar feeders and dependent on nectar sugar to acquire energy for flight activity. Exposure of the adult mosquitoes to the diverse aromatic environment facilitates their learning and maturation of the olfactory system which enables 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

reinforces the mosquito to perform to next level of behavior. Thus, recovery/reactivation of the olfactory responses occurs to find a suitable site for egg laying/oviposition. To capture this molecular snapshot and track the events, we collected olfactory tissues at three different physiological conditions for RNA-Seq 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).

5.2 Material and Methods

Figure 5.2 represents the complete work flow and technical overview of tissue collection, RNAseq mediated transcriptome data generation and the bioinformatics pipeline that was followed for molecular cataloging and functional annotation.

5.2.1 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 fitted with a simulated dawn and dusk machine essentially required for proper mating and feeding. Adult mosquitoes were kept in 1x1x1 $feet^3$ sterile muslin cloth cages, which were sterilized by washing and autoclaving after completion of each colony. A cotton swab dipped into the sterile water was kept on the top of the cages to provide water supplement for the mosquitoes and water soaked raisins were used for sugar supplement.

To maintain the colony of the mosquito species and for the initiation of the gonotrophic cycle, 3-4 days old adult female mosquitoes were allowed to feed on rabbit/mouse obtained from NIMR animal house facility. The gravid females were allowed to lay eggs after 72 hrs. of blood feeding on moistened filter paper which is mounted inside of a small plastic cup (e.g. ice cream cup), that is partially filled with pre-cooled boiled water. Hatched larvae were fed on the mixed dried powder of fish food and dog biscuit. After completion of four larval stages (L1-L4), pupae were collected in a round plastic cup and kept in a mosquito cage that was previously wiped with 70% ethanol. The utensils viz. plastic bowl, cup or tray used to rear larvae were properly washed with soap solution followed by multiple washing with boiled water and air dried. The waste removal and fresh nutrient supply to the larva were maintained with an interval of 24 hrs. in 300-400 ml pre-cooled hot water. To maintain an extra level of sterility adult mosquitoes were fed on sterile sugar solution (5%) using a glass test tube supplied with a sterile cotton swab. All protocols for rearing and maintenance of the mosquito culture were approved by the ethical committee of the institute.

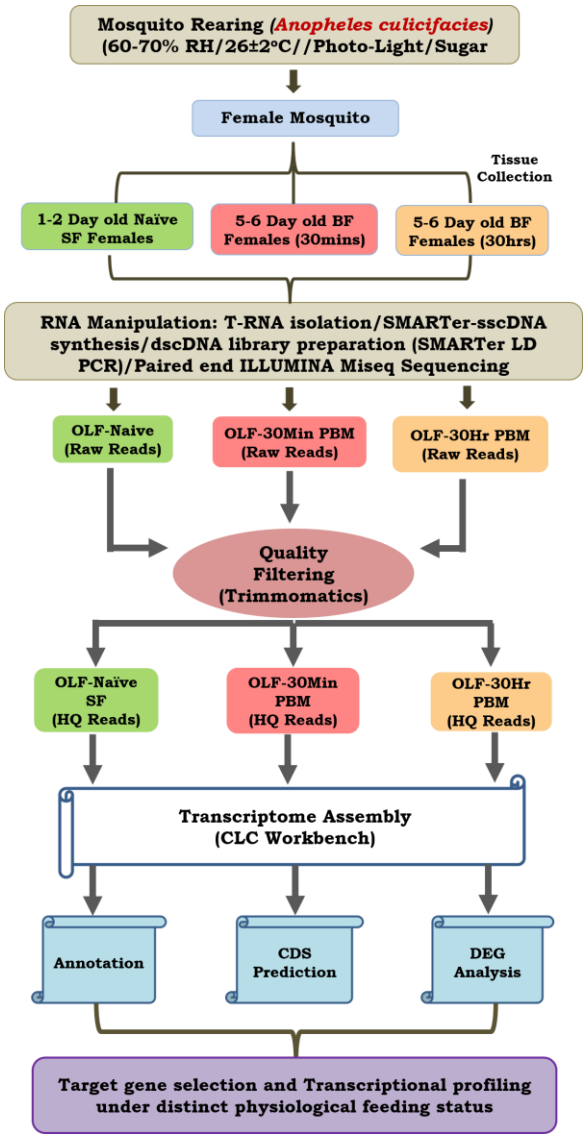

Figure 5.2: A technical overview to decode the hard-wired genetic structure of the olfactory system of *Anopheles culicifacies*. We sequenced and analyzed three RNA samples extracted from the olfactory tissues of approximately 30 mosquitoes individually and pooled to form one single sample.

5.2.2 Tissue Dissection

The olfactory tissues which included antennae, maxillary palp, proboscis, and labium were dissected from cold anesthetized *An. culicifacies* mosquito using entomological needle under dissecting microscope. According to our experimental plan we dissected olfactory tissue from 0-1 day of age, 30min post blood fed and 30 hrs. post blood fed *An. culicifacies* mosquito and collected in Trizol Reagent. Furthermore, different tissues viz. head (male,

female), Legs (male, female), brain (Br), olfactory tissue (OLF), female reproductive organ (FRO) and Male reproductive organ (MAG) of both *An. culicifacies* and *An. stephensi* mosquitoes were dissected and collected in Trizol for gene expression studies.

5.2.3 RNA isolation and transcriptome sequencing analysis

Total RNA was extracted by following the standard Trizol method which includes isopropanol precipitation and ethanol washing [132]. At first, the collected tissue was homogenized by hand-held motorized homogenizer fitted with a detachable autoclaved pestle to disrupt the tissue cells and extract the nucleic acid and protein from the lysed cells. Chloroform was used for the separation of the aqueous layer which contained total RNA. After precipitation, RNA was dissolved in DEPC treated water (15-20 µl) (80˚C) and measured its concentration by Nano-drop 2000 spectrophotometer (Thermo Scientific). RNA was stored at -80˚C after addition of RNase inhibitor (Ribolock, Thermoscientific).

5.2.4 cDNA library preparation

Approximately 1ug total RNA was used for reverse-transcription reaction to prepare single-stranded cDNA (sscDNA) using SMARTer PCR cDNA synthesis kit (catalogue No. 634925; BD Clontech, Palo Alto, CA, USA) by following the protocol as described by the manufacturer. The quality of sscDNA synthesis was assessed by PCR amplification of ribosomal protein S7 gene or Actin gene [19]. After quality assurance, double-stranded cDNA (dscDNA) were prepared from the sscDNA through LD-PCR protocol using Advantage 2 PCR kit (Dixit, Rawat et al. 2011). Then dscDNA was purified using Fermentas GeneJET Extraction kit (#K0691) and eluted in 25-30 µl sterile DEPC water. The quantity of purified dscDNA was examined by nanodrop and outsourced the respective samples for transcriptome sequencing. The quality of dscDNA was checked on Caliper LabChip GX using HT DNA High Sensitivity Assay Kit (Agilent) as per manufacturer's instructions.

5.2.5 Illumina MiSeq 2X150 PE library preparation and sequencing

The pair-end cDNA sequencing libraries were prepared using Illumina TruSeq RNA Library Preparation Kit as per the protocol mentioned. For library preparation, the following steps are followed (i) shearing of dscDNA followed by end repair, (ii) adapter ligation and (iii) index PCR amplification of adaptor-ligated library. Again library quantification and qualification were performed on Caliper LabChip GX using HT DNA High Sensitivity Assay Kit and the next generation sequencing was performed on Illumina MiSeq resulting an output of RNA-Seq reads.

5.2.6 RNA-Seq database annotation and molecular cataloguing

Millions of RNA-Seq raw reads were filtered using Trimmomatic v0.30 software to remove the adaptor contamination and the reads with low-quality value. These clean reads were further processed for de-novo assembly using CLC genomics workbench on default parameters (Minimum contig length: 200, Automatic word size: Yes, Perform scaffolding: Yes, Mismatch cost: 2, Insertion cost: 3, Deletion cost: 3, Length fraction: 0.5, Similarity fraction: 0.8) to make the contigs. To predict the coding nature of the olfactory transcripts, the assembled contigs were further processed using the online available Transdecoder software. For a comprehensive functional annotation of the predicted transcripts, an integrated bioinformatic pipeline was followed which included mapping with multiple protein databases such as non-redundant (NR) database from NCBI, GO, SMART, PFAM etc. Finally, differential gene expression analysis was done by DESeq R package software to represent the differentially expressed genes among different samples. The sequencing data were deposited to the National Center for Biotechnology Information (NCBI) Sequence Reads Archive (SRA) system (BioProject accessions: PRJNA414162; BioSample accessions: SAMN07981002, SAMN07972755, and SAMN07775994).

5.2.7 Primer designing

To design the primers for PCR amplification of selected transcripts, online Primer3 software (http://bioinfo.ut.ee/primer3/) was used. The default parameters of the software were used except the product size, which was kept in a range of ~200-300bp length, preferring location towards the 3' end of the gene of interest. Both forward and reverse primer sequences were outsourced for synthesis. Following is the list of primers sequences that were used in the current work (Table 5.1).

Table 5.1: List of primers and their sequences

Serial No.	Primer Name and Sequence
1.	OBP5_Fw: 5'CGGGAACTAAAATGCTACAC 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' GGCGGTATCACATTTATCCGG 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'GGGTTCCTATTTGTGGTCGG3' 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' CTTCATTTGTCGGTCTTGAT 3' Or44_Rev: 5' CCTCAAAGAACTTGCGATAC 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' TTTAGGTTGTTCCTGCAGTC 3' Uncharacterized Protein: 5' ACTGATGTGAAGCAGAATCC 3'
22.	Or9_Fw: 5' AGATTGCCTACAACTTCACC 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’

5.2.8 PCR based Gene Expression Analysis

5.2.8.1 cDNA Synthesis

Approximately 1µg total RNA was used to synthesize single-stranded cDNA using Verso cDNA synthesis kit as per the manufacturer’s protocol (ThermoScientific, Catalogue # AB-1453/A). Initially, DNA free 1 µg template RNA was subjected to heat at 70˚C for 5 minutes to denature any secondary structure present in RNA. Then, the reaction mixture was added which was prepared using 5X cDNA synthesis buffer (final concentration 1X), 500 µM dNTP mix, 1 µL of RNA Primer (OligodT and random hexamer), RT Enhancer, Verso Enzyme Mix. Finally, total volume was adjusted to 20 µl with PCR grade sterile water. Reverse transcription was conducted at 42˚C for half an hour and ceased at 95˚C for 2 minutes to yield a quality cDNA template for gene expression analysis.

5.2.8.2 RT-PCR

Differential gene expression analysis was performed using the normal Reverse Transcription-polymerase chain reaction (RT-PCR) and agarose gel electrophoresis protocol. 10 µl reaction mixture was used for each PCR reaction which contained 1X PCR master mix (EmeraldAMP GT PCR Master Mix 2X), 0.8 pmol/ µl forward and reverse primer each, ~50ng template cDNA and volume make up was done by water. The PCR amplification was done by Benchtop PCR machine (Benchtop lab system thermocycler) by following the PCR cycle parameters: Initial denaturation at 95˚C for 5 min, followed by 30 cycles: 95˚C for 30 seconds (denaturation), 52˚C for 30 seconds (annealing), 72˚C for 30 seconds (amplification), and final extension step at 72˚C for 5 minutes. Amplified PCR products were resolved on 1.5% agarose gels (HiMedia) and product sizes were calculated by comparing with marker fragments of 100bp DNA ladder (Invitrogen, USA).

5.2.8.3 Real-Time PCR

For relative gene expression analysis, we used SYBR green qPCR (Thermo Scientific and Kapa Biosystem) master mix and Illumina Eco Real-Time PCR machine, as described earlier [22]. PCR cycle parameters involved an initial denaturation at 95ºC 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 secs and again 95°C for 15 secs 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 was used as internal control in all the experiment and the relative quantification was analyzed by $2^{-\Delta\Delta Ct}$ method [133]. Differential gene expression was statistically analyzed using student *'t'* test.

5.2.9 Blood meal time series follow up

For blood meal time series follow up experiment, the olfactory tissues were collected from both naïve sugar fed and blood fed mosquitoes at different time points. For each time point, the olfactory tissues were dissected from at least 25-30 adult female mosquitoes and pulled in a single tube. 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 the 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 was done after 30hrs of 2nd blood meal (Figure 5.3).

5.2.10 Structural modeling and functional prediction analysis of SAP1 and SAP2 protein

For structure prediction analysis of SAP1 and SAP2 proteins from *An. culicifacies,* initially, a template for each query proteins were searched against PDB database using BLASTP algorithm. Two best templates were selected for each used query sequence and thereafter, modeller9v.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.

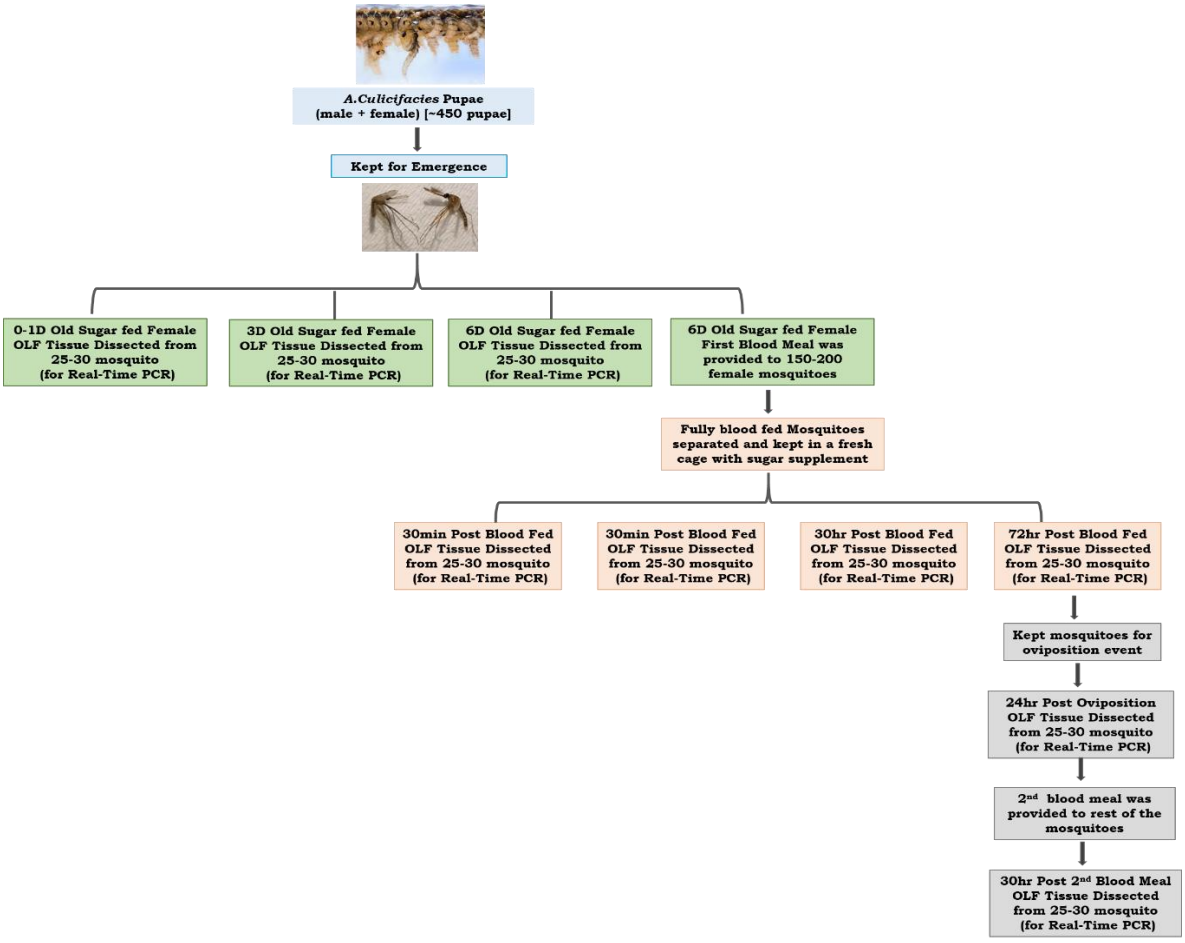

Figure 5.3: Pictorial presentation of blood meal time series experiment.

5.3 Results and Discussion

5.3.1 RNA-Seq database generation, assembly, and annotation

To unravel the molecular nature of olfactory management process during pre and post blood meal conditions, a total of ~122 million RNA-Seq reads were generated from the olfactory tissues collected from 1-2 day old naive (Nv), 5-6-day old immediate blood fed (30m-2h PBM) and 30hr post blood fed (30hr PBM) mosquitoes. We chose 30h PBM as a critical time when completion of blood meal digestion occurs in the midgut, possibly which may have direct influence on the reactivation of the olfactory system [126,127,134] (Figure 5.4). Each RNA-Seq database was assembled into contigs/Transcripts and compared against multiple molecular databases for molecular and functional annotation analysis of each transcript. Our initial attempt of mapping cleaned reads to the available draft reference genome failed to yield quality results, probably due to the poor annotation of the *An. culicifacies* genome. Alternatively, we performed a *denovo* assembly to construct

a molecular map of the mosquito olfactory system. Table 5.2 represents details of the annotation kinetics of mosquito olfactory databases.

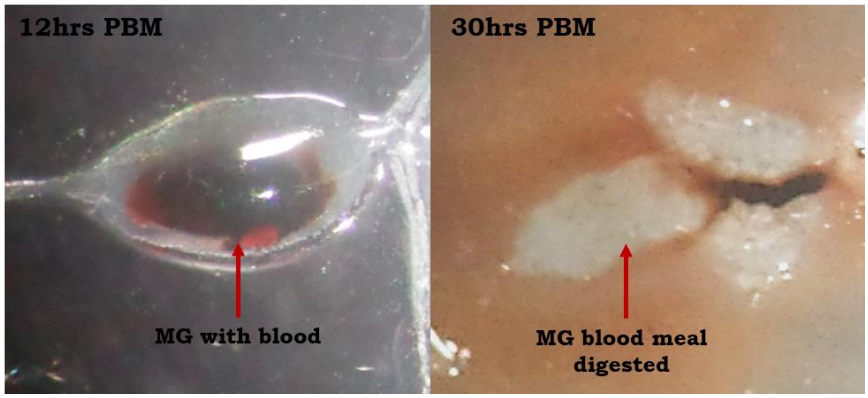

Figure 5.4: Time-dependent depiction of the blood-fed midgut (MG) showing blood meal digestion. MG digested from 12hrs post blood meal (PBM) showed undigested blood still present in the MG but after 30hrs of PBM blood meal digestion completed and thus no blood remnants is observable.

Table 5.2: Annotation kinetics of the RNA-Seq data

Molecular Features	Ac-OLF-Naive	Ac-OLF-30M PBM	Ac-OLF-30Hr PBM
Total number of Reads	4001428	4435700	3841295
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%)

5.3.2 Molecular composition and key changes in response to blood meal

To test how blood meal, affect the global expression pattern of the olfactory transcriptome, at first we performed a digital gene expression (DGE) analyses using DESeq R package software as described earlier [19]. Our analysis revealed that blood meal causes a modest shift in the transcriptome expression (Figure 5.5a), supporting the previous observations that first blood meal enhances odorant receptor transcripts abundance modestly, but causes

a general reduction of mosquito antennal chemosensory gene repertoire in *An. gambiae* [127].

A comprehensive analysis further revealed 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 5.3). 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 5.3).

Table 5.3: 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

Furthermore, a GO-based functional annotation and comparative analysis revealed that the basic composition of the mosquito olfactory tissue does not alter significantly (Figure 5.5b-d) in response to blood feeding. But, the response to stimulus and the signaling category of genes showed a difference in their abundance when compared to naïve vs blood fed conditions (Figure 5.5b-d). Observation of a limited change in the olfactory responses allowed us to 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 a stimulus, circadian rhythm, and signaling in the blood fed adult female mosquitoes. To clarify this complexity, first, we manually shortlisted the olfactory transcripts either based on their FPKM abundance and/or predicted coding nature. To further predict the possible molecular link, a set of unique genes were analyzed through extensive profiling of their transcriptional regulation which may likely to influence mosquito host-seeking and blood-feeding behavior.

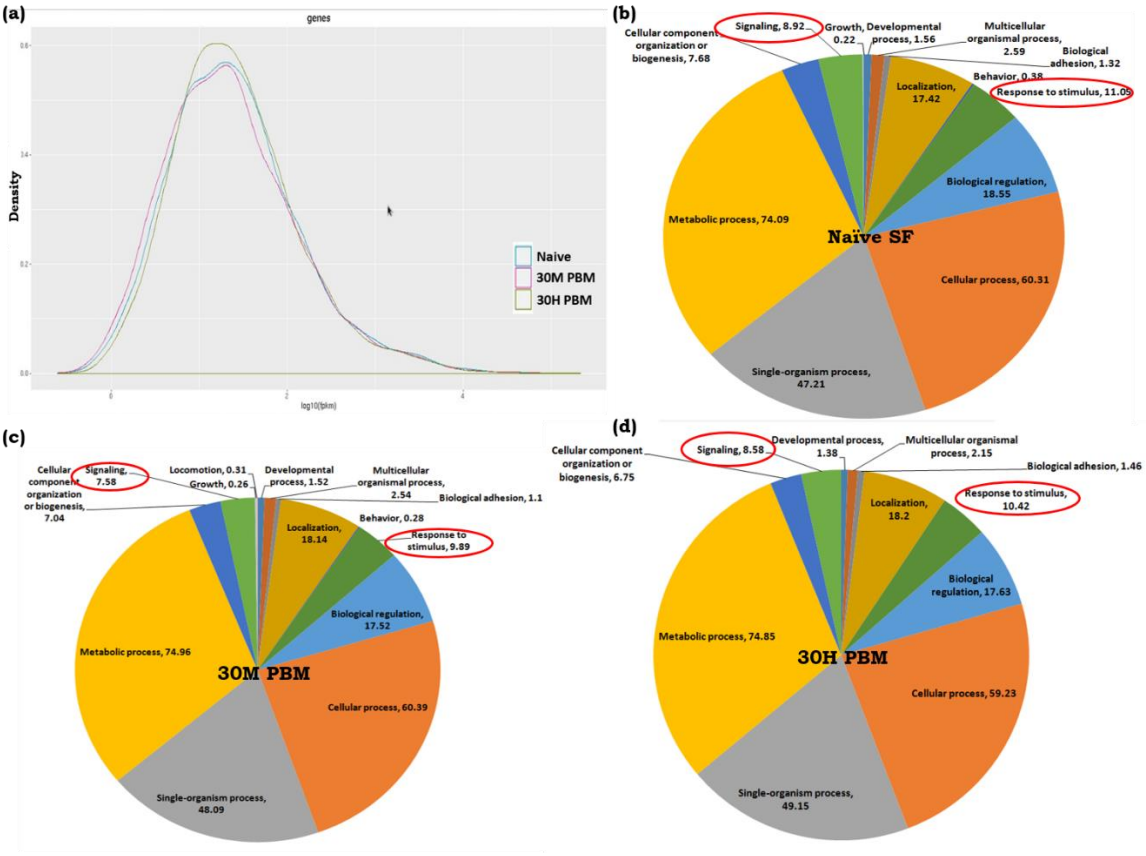

Figure 5.5: Blood meal cause modest changes in the molecular architecture of the mosquito olfactory system. (a) Read density map of the compared Naive; 30M and 30Hr post blood meal (PBM) transcriptomic data of olfactory system; (b-d) Functional annotation and molecular cataloguing of the olfactory transcriptome (Biological Process/Level2/% Transcripts). Red circle marks the genes selected for transcriptional response monitoring (See Text).

5.3.3 Molecular analysis of the chemosensory gene repertoire

The management of navigation trajectory towards the vertebrate host and each successful feeding event are strictly regulated by the olfactory encoded proteins of diverse nature which includes a soluble carrier protein, membrane receptors and degrading enzymes. Among them, odorant binding proteins (OBPs) play a primary and crucial role to bind and deliver the odorants/chemicals information to their cognate odorant receptors. After binding of the odorant molecule to their respective receptors, the actual signal transduction procedure started which then convey the initial signal of odor to higher brain centers, where behavioral decisions making procedure occurred. Thus, to decode the complex path of odor detection mechanism, a detailed cataloguing and functional analysis of Odorant Binding Proteins (OBPs) and Olfactory Receptors (Ors) genes were performed in this studies.

5.3.3.1 Molecular cataloguing of Odorant Binding Proteins (OBPs)

To make a comprehensive molecular catalog of the Odorant Binding Protein Family, first, we extracted all the OBP transcript present in our transcriptome database by performing extensive BLAST homology search analysis against multiple insect OBP databases. Next, we also predicted and annotated more OBP genes from the genome database of *An. culicifacies*, available in www.vectorbase.org by using the previously extracted OBP transcript as a reference. Figure 5.6 represents the technical workflow used for the retrieval and cataloguing of the olfactory OBPs.

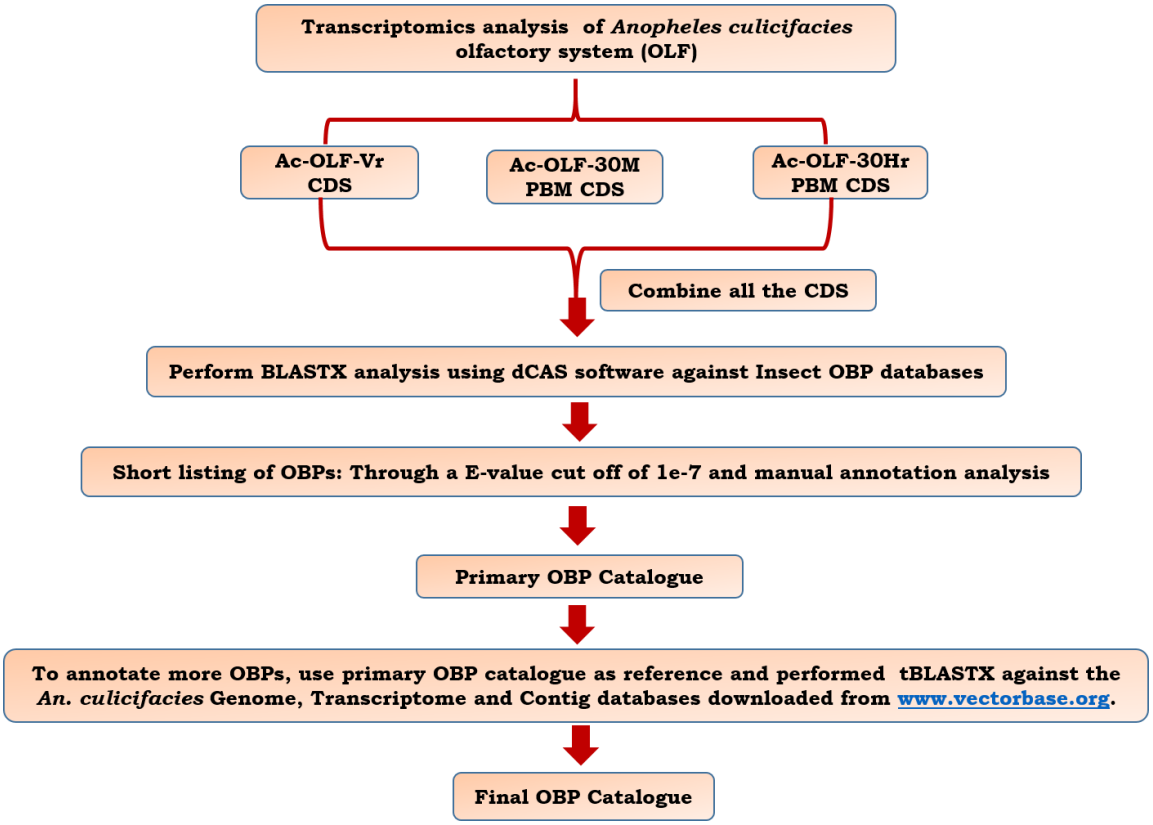

Figure 5.6: Schematic presentation of retrieval of OBP from transcriptome and genome data of *An. culicifacies*

Homology search analysis coupled with the Desktop cDNA Annotation System (dCAS) software-mediated bioinformatics analysis against draft genome allowed the identification of total sixty-three OBP genes from the mosquito *An. culicifacies.* Further annotation and classification of all the 63 OBPs were done by manual analysis for the presence of specific characteristic features of each group of the OBPs (Table 5.4).

Table 5.4a: List of OBP genes annotated from the olfactory transcriptome data
(*Newly annotated OBPs)

Sl. No.	Transcript ID	Origin of the Transcript	Name	*An. culicifacies* Database Match	Sub-Family	Ortholog in *An. gambiae*	FPKM
1.	Transcript_1018	30hr PBM	OBP20	ACUA000236	Classic	AGAP005208	3173.278225207 92
2.	Transcript_1216	30hr PBM	GOBP72	ACUA019958 *	Classic	AGAP012714	1254.82586
3.	Transcript_127	30hr PBM	OBP7	ACUA012847	Classic	AGAP001556	4144.93009
4.	Transcript_1450	30hr PBM	OBP26	ACUA003587 *	Classic	AGAP012321	2051.81759
5.	Transcript_4885	30hr PBM	OBP63	ACUA020502 *	Classic	AGAP012322	187.690564
6.	Transcript_6613	30hr PBM	OBP3	ACUA024106	Classic	AGAP001409	21144.6696
7.	Transcript_91	30hr PBM	GOBP71	ACUA003567	Classic	AGAP012331	4939.85295
8.	Transcript_109	Naive	OBP5	ACUA003763	Classic	AGAP009629	301.156325
9.	Transcript_3113	Naive	OBP28	ACUA001691	Classic	AGAP012325	63.5528511
10.	Transcript_1290	Naive	OBP9	ACUA003296	Classic	AGAP000278	389.876797
11.	Transcript_138	Naive	OBP6	ACUA004501 *	Classic	AGAP003530	238.355131
12.	Transcript_270	Naive	OBP2	ACUA004189	Classic	AGAP003306	211.717742
13.	Transcript_218	30min_PBM	OBP10	ACUA017068	Classic	AGAP001189	40.2405414
14.	Transcript_48	30min_PBM	OBP25	ACUA014063	Classic	AGAP012320	92.3071105
15.	Transcript_62	30min_PBM	OBP1	ACUA014299	Classic	AGAP003309	608.296339

16.	Transcript_59	30min_PBM	OBP54	ACUA001499 *	Pluc-C	AGAP006080	41.4381766
17.	Transcript_3357	30min_PBM	GOBP69	ACUA024897	Pluc-C	AGAP013182	323.549283
18.	Transcript_207	30min_PBM	OBP47	ACUA011874	Pluc-C	AGAP007287	116.931
19.	Transcript_7419	30min_PBM	OBP58	ACUA019212 *	Pluc-C	AGAP006074	28.093679
20.	Transcript_5393	30min_PBM	OBP46	ACUA020810	Pluc-C	AGAP007289	23.4694097
21.	Transcript_6620	30min_PBM	OBP56	ACUA025325	Pluc-C	AGAP011367	20.576198
22.	Transcript_611	Naive	OBP57	ACUA028407	Pluc-C	AGAP011368	1983.04862
23.	Transcript_4488	Naive	OBP51	ACUA023815 *	Pluc-C	AGAP006077	65432.854
24.	Transcript_22	Naive	OBP48	ACUA025211	Pluc-C	AGAP007286	1728.56394
25.	Transcript_853	30hr PBM	OBP43	ACUA001907	Two Domain	AGAP009402	90.5977525
26.	Transcript_2542	30hr PBM	D7	ACUA025281 *	D7 Protein Family	AGAP006278	462.002781
27.	Transcript_2682	30hr PBM	CSP	ACUA008309 *	Chemosensory Protein	AGAP001303	215.86399
28.	Transcript_5645	30hr PBM	LD7	ACUA002223	D7 Protein Family	AGAP007286	34824.3385
29.	Transcript_64	30hr PBM	SD7	ACUA001093	D7 Protein Family	AGAP008281	1348.33451
30.	Transcript_1808	Naive	CSP	ACUA010232 *	Chemosensory Protein	AGAP008055	519.825389
31.	Transcript_447	Naive	CSP3	ACUA004458	Chemosensory Protein	AGAP008059	195.413717
32.	Transcript_702	Naive	CSP	ACUA017151	Chemosensory Protein	AGAP008054	203.377554

33.	Transcript_12	Naive	Sensory Appendage Protein	ACUA018714	Chemosensory Protein	AGAP008051	24346.8834
34.	Transcript_5005	Naive	LD7	ACUA018525	D7 Protein Family	AGAP008279	53.6723049
35.	Transcript_631	Naive	LD7	ACUA008578	D7 Protein Family	AGAP028120	27.625451
36.	Transcript_1446	30min_PBM	CSP	ACUA013563 *	Chemosensory Protein	AGAP008058	149.776542

Table 5.4b: List of OBP genes that are annotated in the genome

Serial No.	*An.culicifacies* Transcript ID	Name	Signal Peptide	OBP Subfamily	Ortholog in *An.gambiae*
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

Domain prediction analysis classified the OBPs as Classic OBPs, Plus-C OBPs, Two-domain OBPs and other chemosensory protein family, as described earlier for the mosquito *An. gambiae* [56]. A multiple sequence alignment and phylogenetic analysis of all the OBP classes indicated that as compared to Plus-C and Atypical OBPs (Figure 5.7 and 5.8), the molecular signatures of Classic-OBPs are more conserved (Figure 5.9) and widely present within the insects' taxa including *Drososphila* sp. Whereas, Plus-C OBPs and Atypical OBPs seem to be unique to the mosquitoes suggesting their possible involvement in the evolution and adaptation of blood feeding behavior of adult female mosquitoes

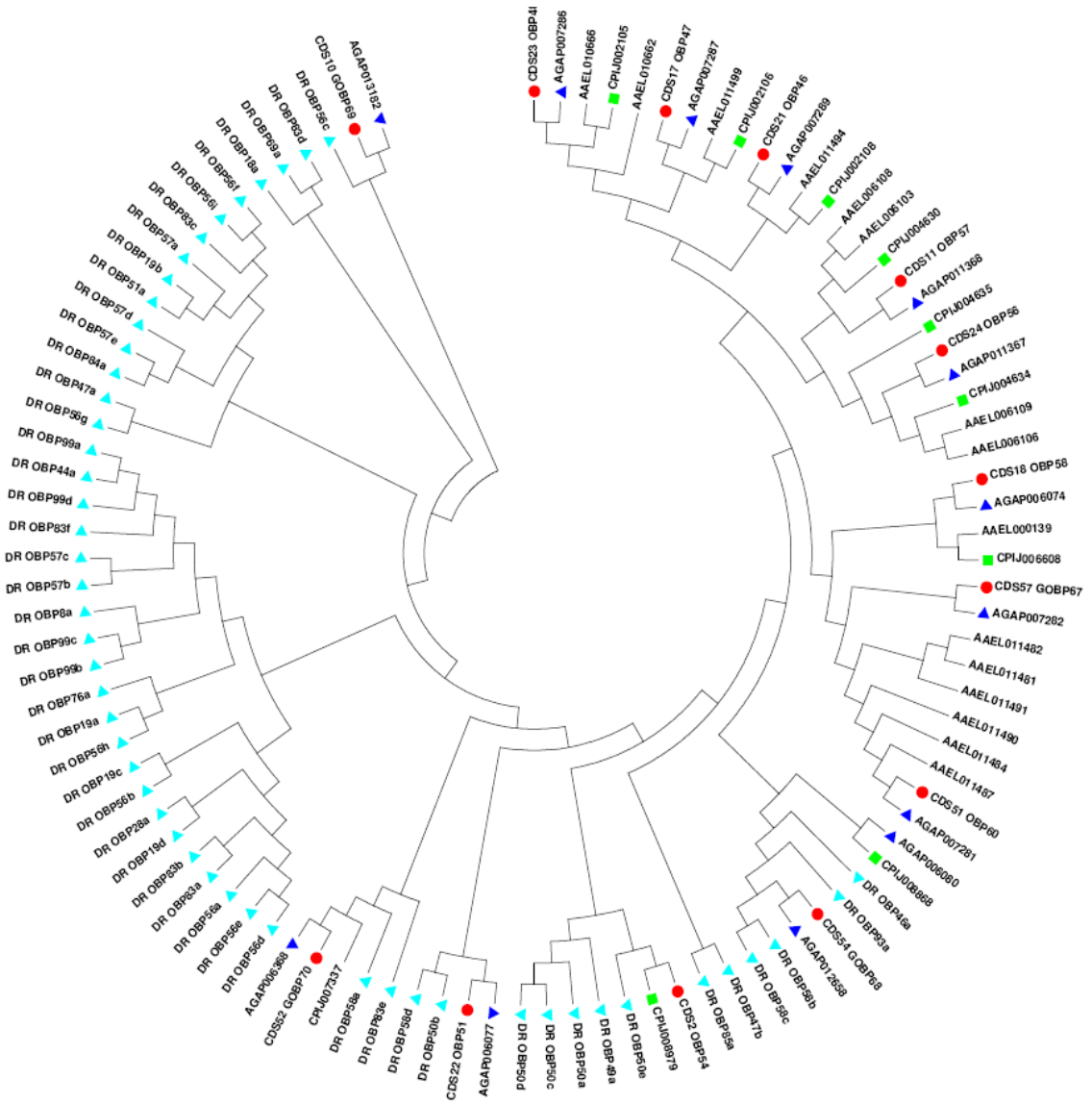

Figure 5.7: Phylogenetic analysis of Plus-C OBP superfamily. *An. culicifacies* Plus-C class of OBPs are more closely linked to mosquito specific OBPs and showed distant relationship to other non-mosquito species for example *Drosophila*. Different color code that indicating a particular mosquito and 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.

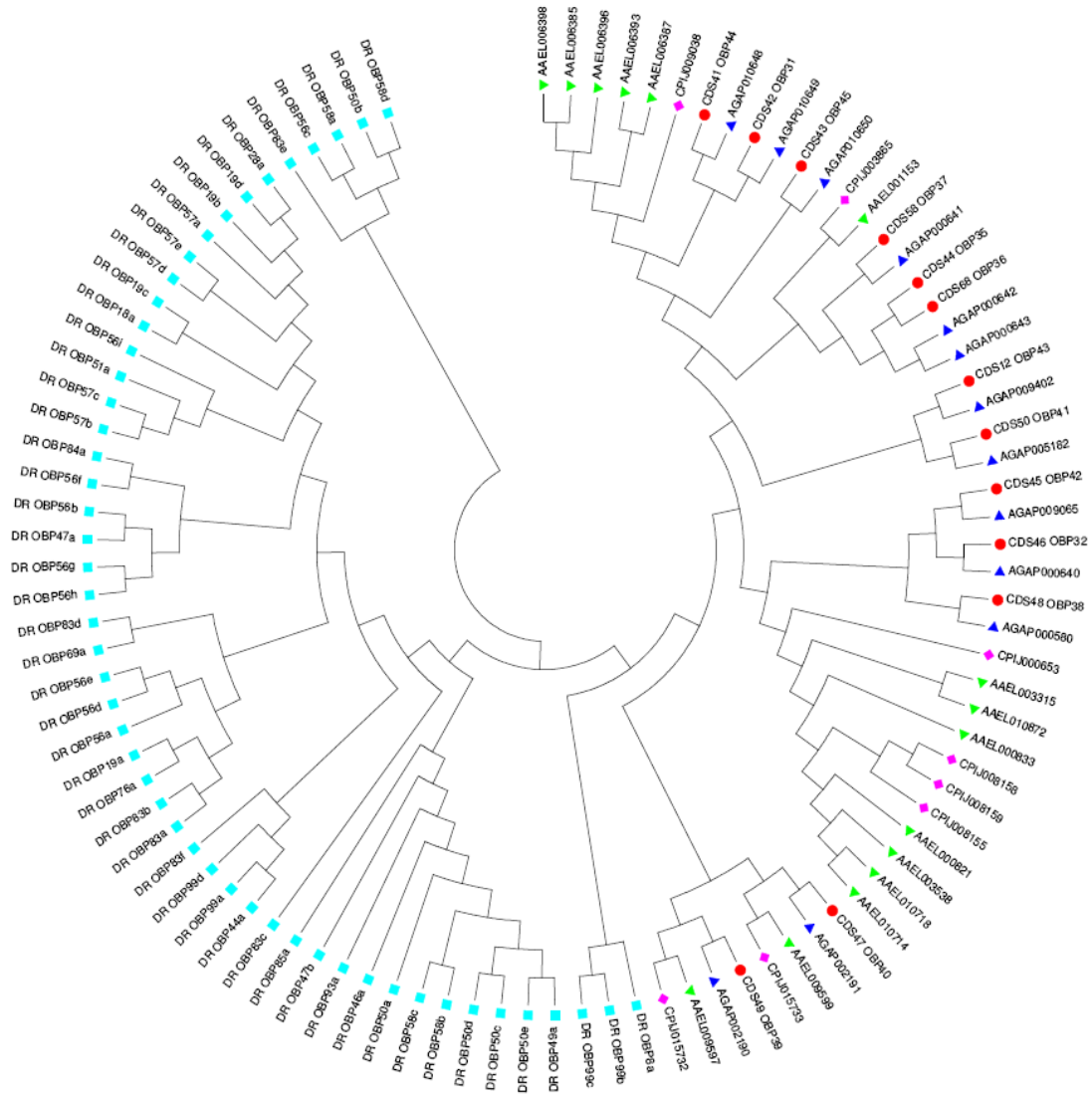

Figure 5.8: Phylogenetic analysis of Atypical OBP superfamily. *An. culicifacies* Atypical OBP class are more closely linked to different mosquito species and showed distant relationship to other non-mosquito species for example *Drosophila.* Different color code that indicating a particular mosquito and insect species: Blue – *An. gambiae*; Red – *An. culicifacies*, Pink – *Culex quinquefasciatus*; Sky blue – *D. melanogaster;* Green - *Aedes aegypti*.

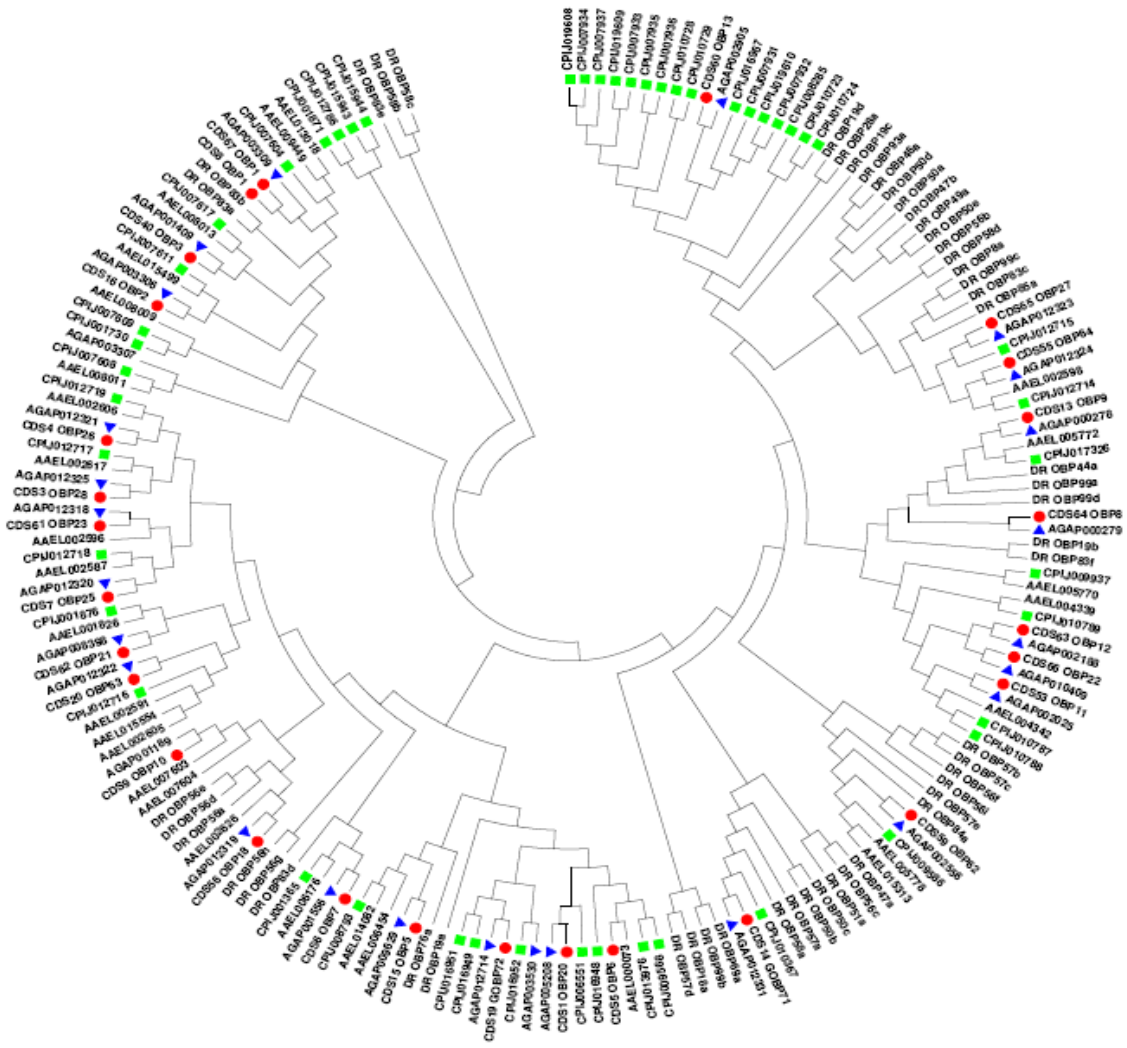

Figure 5.9: Phylogenetic analysis of Classic-OBPs. Classic-OBPs of *An. culicifacies* showed conserved sequence relationship with *An. gambiae* and other mosquito and insect species. Different color code that indicates a particular mosquito and insect species: Blue – *An. gambiae*; Red – *An. culicifacies*, Green – *Culex quinquefasciatus*. *Drosophila melanogaster* (DRX) and *Aedes aegypti* (AAX) are not marked with any color code.

5.3.3.2 Daily rhythmic expression analysis of Odorant binding proteins (OBPs)

Our observation of DGE data indicated that first blood meal restricted the expression of common OBP transcripts (Figure 5.10a), but causes the appearance of unique OBP transcripts (Table 5.4), possibly to manage crucial event in modulating the behavioral activities in response to changing in the feeding status from naive sugar feeding to blood feeding. To further validate and unravel this unique relationship of OBPs regulation, the expression of at least 15 putative OBP transcripts were examined under distinct conditions. In this analysis, two uncharacterized chemosensory proteins (CSPs) named sensory

appendage protein (SAP1 & SAP2) were also included which showed a dominant expression in the naive mosquito olfactory tissue (Table 5.4a). Previous literature suggested that the molecular signature of host-seeking behavioral property resides within those olfactory genes that showed dynamic expression pattern according to the circadian rhythm [135]. Thus, to unravel the molecular basis of host finding associated behavioral complexity in *An. culicifacies* mosquito, we performed a relative expression profiling of the OBP genes in the olfactory tissue, dissected at different zeitgeber time point (Figure 5.10b). The 24 hrs time scale of the Light-Dark (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.

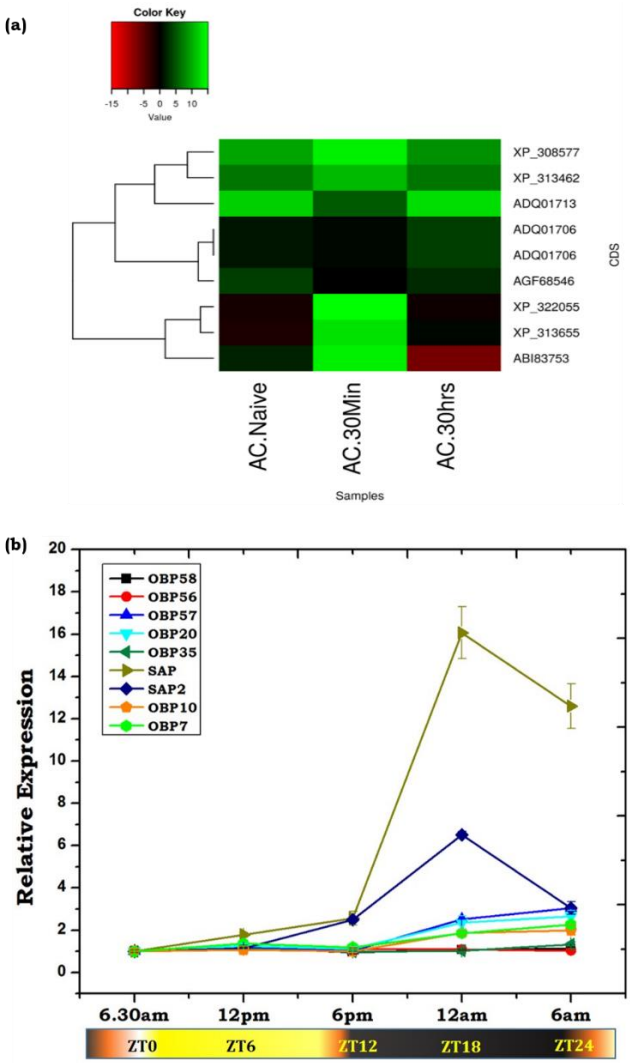

Figure 5.10: Transcriptional response of OBP genes. (a) Heat map showing differential expression pattern of eight common OBP genes in naïve and blood fed olfactory tissue of *An. culicifacies*. (b) Rhythmic expression of OBP genes in the adult female's olfactory tissues (OLF) according to different zeitgeber 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.

Zeitgeber time scale expression revealed 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 (Figure 5.10b). These data also corroborate with the previous observation of the natural active biting behavior of *An. culicifacies* mosquito in the midnight [136,137]. 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.

5.3.3.3 Blood meal modulate OBPs gene expression

A significant modulation of the OBP genes expression according to the day-night cycle reinforce us to study its expression pattern depending on the feeding status of the mosquitoes. To evaluate the role of OBP genes in regulating and managing the differential feeding behavior, a blood meal time series experiment was performed, where the olfactory tissues were dissected from naïve sugar fed and blood fed mosquitoes. A transient suppression (30min) and rapid recovery of OBPs expression were observed as a result of intake of first blood meal (Figure 5.11).

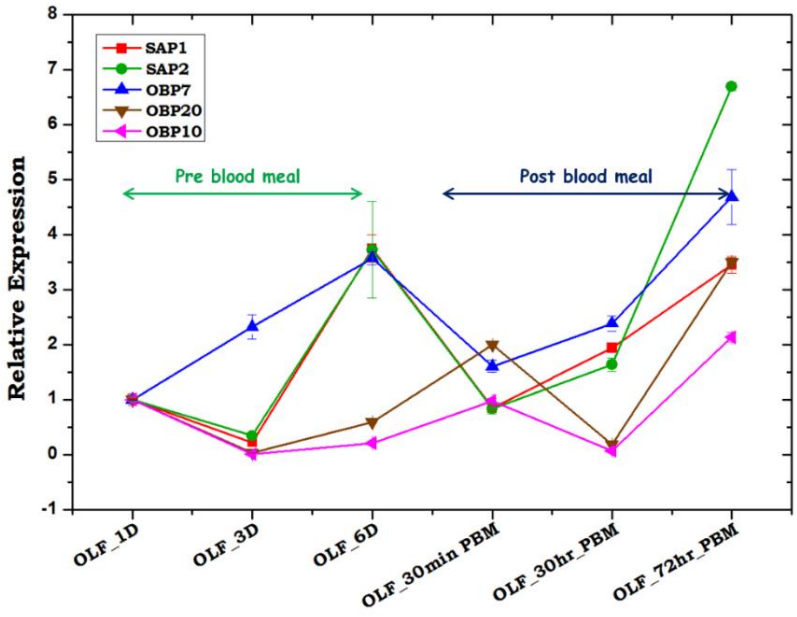

Figure 5.11: Relative expression profiling of OBP genes in pre and post blood fed olfactory tissues. Olfactory tissues (OLF) were collected from 1day, 3day and 6day old sugar-fed mosquitoes which were then provided blood meal and then the olfactory tissues were collected after 30mins of post blood fed 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.

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 that 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.

5.3.3.4 Molecular cataloguing and analysis of Olfactory Receptors (Ors)

A significant 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, a total of 603 unique transcripts linked to response to stimulus and signaling (RTSS) categories were retrieved, pooled and catalogued (Figure 5.12a), which encoded diverse nature of proteins such as anion binding, nucleic acid binding, receptor activity, hydrolases, and transferase activity (Figure 5.12a). Surprisingly, out of 603 transcripts, greater than 100 transcripts were found to uniquely associated with individual physiological conditions compared in the study while ~110 transcripts were common to all (Figure 5.12b). 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.

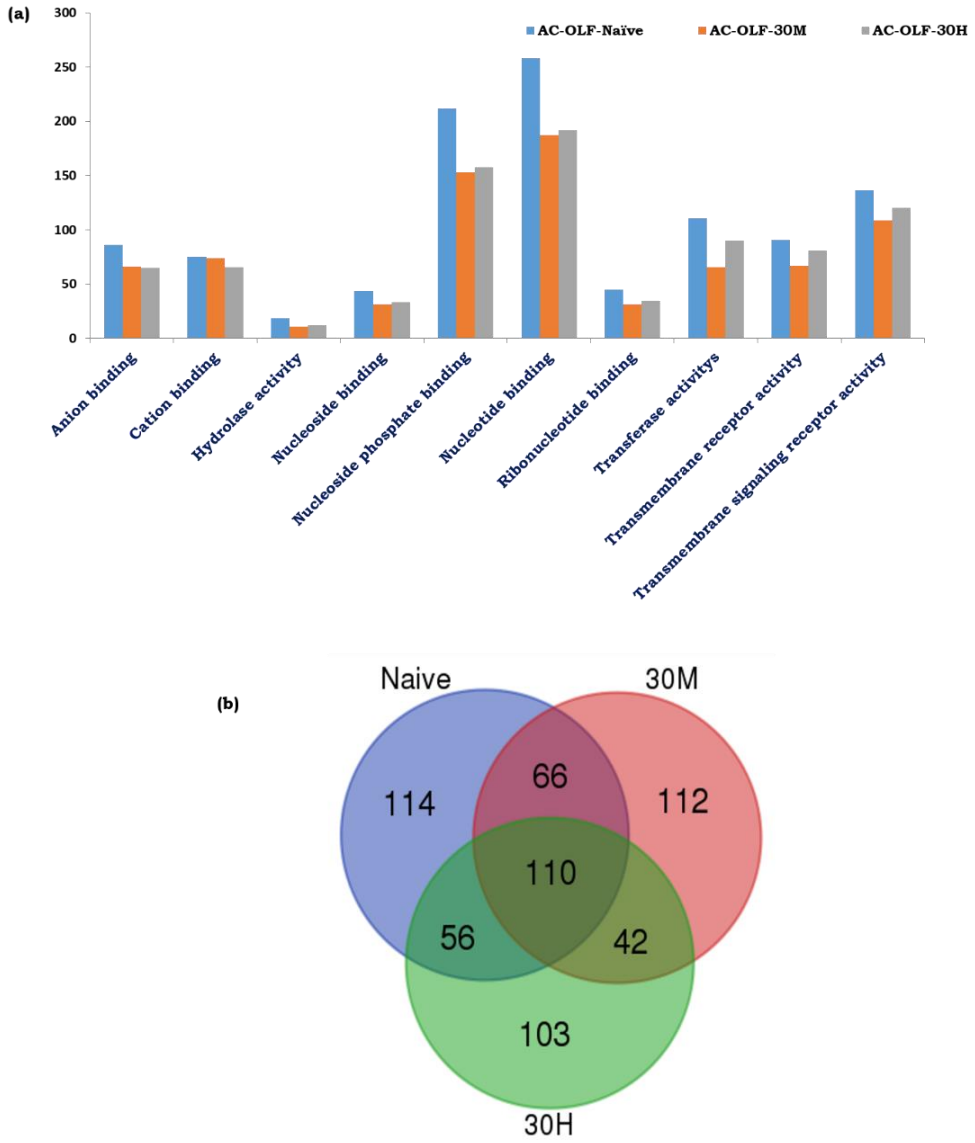

Figure 5.12: Detail molecular cataloguing of response to stimulus and signaling category of genes. (a) A comparative GO score distribution analysis of the response to stimulus and signaling transcripts of naïve and blood-fed mosquitoes. (b) Venn diagram showing common and unique transcripts of response to stimulus and signaling GO category of naïve and blood-fed mosquitoes.

Mosquito olfactory receptors are thought to play a central role to receive and communicate the chemical message to higher brain center through olfactory receptor neurons for decision making events [33,138,139]**.** To further clarify this molecular relation, initially we performed a manual analysis of the RTSS category, catalogued 50 different chemosensory receptor genes (Table 5.5a), which comprise odorant receptors (Ors); gustatory receptors (Grs) and variant ionotropic receptors, through manual analysis of the RTSS category (Table 5.5b).

Interestingly, a cluster of 19 different olfactory receptor genes was found to be expressed abundantly and exclusively in the naïve mosquito (Table 5.5b). However, 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 5.5b). Observation of the constitutive expression of Orco and few other Ors and Grs (totaling 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 to blood fed status.

Table 5.5a: Olfactory Receptor Transcripts retrieved from each sample

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

Table 5.5b: Catalogue of Olfactory Receptors in *Anopheles culicifacies* mosquito:

Sl No.	Gene Accession No.	Receptor Type	*An. culicifacies* ID	*An. gambiae* Identity	CDS Origin	FPKM
1.	XP_310061	Or29	ACUA025107	AGAP009111	Naïve_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	14.690214 5
5.	XP_311894	OR62	ACUA008089	AGAP011978	Naïve_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	Naïve_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	ACUA019523	AGAP011991	30hr PBM_Transcript_3929	16.2275377
32.	EFR26255	Putative GPCR class an orphan receptor 21	ACUA001749	AGAP005681	30hr PBM_Transcript_5866	5731.48201
33.	XP_309588	Putative GPCR class an orphan receptor 4	ACUA021279	AGAP004034	30hr PBM_Transcript_8157	1696.81659
34.	XP_320564	IR76b	ACUA015047	AGAP011968	30hr PBM_Transcript_4542	48.3445393
35.	EFR26426	IR76a	ACUA001357	AGAP004923	30hr PBM_Transcript_7611	21.7380598
	Common Receptor Genes expressed in all the experimental conditions.					

36.	ACQ55870	Or 45	ACUA019417	AGAP003053	30hr PBM_Transcript_4029	19.0208024
37.	XP_556129	Gustatory Receptor	ACUA021260	AGAP005495	Naive_Transcript_2080	7531.73118
38.	XP_320553	Or62	ACUA008089	AGAP011978	Naive_Transcript_1886	2125.35094
39.	ABK97614	Gr24	ACUA010490	AGAP001915	Naive_Transcript_7268	661.574914
40.	XP_312379	Orco	ACUA003469	AGAP002560	Naive_Transcript_972	425.790389
41.	XP_320874	Or11	ACUA013073	AGAP011631	Naive_Transcript_3225	341.520417
42.	XP_319142	Gr22	ACUA004054	AGAP009999	Naive_Transcript_1868	148.739849
43.	ACS83758	Or66	ACUA000316	AGAP003310	Naive_Transcript_5257	66.5066074
44.	XP_312203	Or28	ACUA009796	AGAP002722	Naive_Transcript_2977	47.830094
45.	XP_314480	Or24	ACUA011915	AGAP010507	Naive_Transcript_6099	78.408311 3
46.	XP_001688726	Or33	ACUA020367	AGAP005760	Naive_Transcript_3900	25.280289 4
47.	XP_307763	Gr17	ACUA019767	AGAP003255	Naive_Transcript_7167	25.232859 2
48.	XP_319861	Or29	ACUA025107	AGAP009111	Naive_Transcript_5446	53.317533 4
49.	XP_321153	Or8	ACUA014383	AGAP001912	30minPBM_Transcript_1795	259.15027 7
50.	XP_313200	Or42	ACUA024848	AGAP004278	Naive_Transcript_7008	18.125490 5

5.3.3.5 Rhythmic expression of Olfactory Receptors

To understand the coordinated expression pattern of the olfactory receptors with OBPs in response to circadian rhythm, we performed olfactory receptor expression profiling at the same zeitgeber time point, similar to OBPs expression analysis. Unlike OBPs, poor modulation of olfactory receptor gene expression under circadian rhythm (Figure 5.13) 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 monitor the transcriptional behavior of the selected Ors transcripts in response to two consecutive blood meal series follow-up.

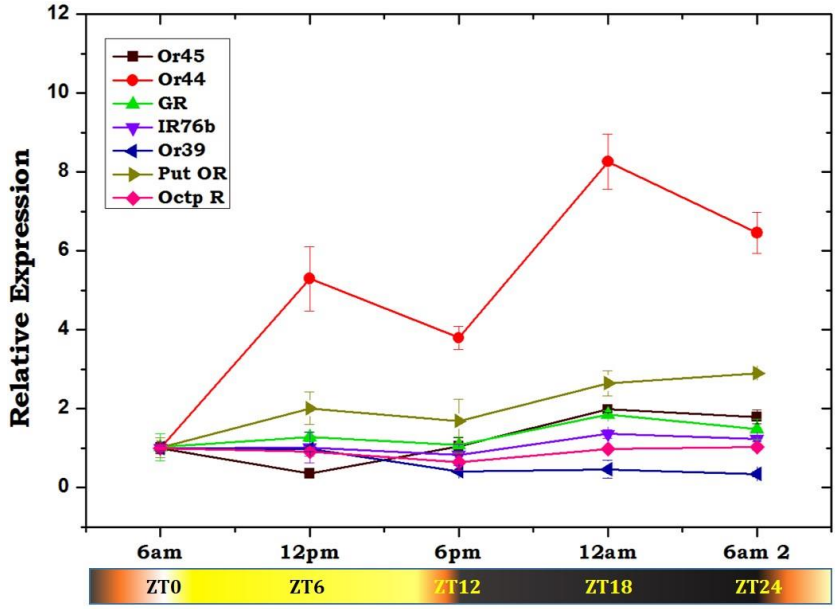

Figure 5.13: Rhythmic expression of olfactory receptor genes (Ors) of *An. culicifacies* in the olfactory tissues of female mosquitoes, where ZT0 indicate the end of dawn transition, ZT11 is defined as the start of the dusk transition and ZT12 is defined as the time of lights off.

5.3.3.6 Innate physiological status modulate olfactory receptor responses

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 [140–142]. 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 [127,143,144]. Thus, 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 unraveled.

To further corroborate with the above propositions and uncover the possible functional correlations of olfactory receptor responses, the transcriptional behavior of the selected Ors transcripts was monitored in response to two consecutive blood meal series follow-up experiment. An age-dependent enrichment of Ors transcripts till 6th day of maturation in

the naïve 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 (Figure 5.14).

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 (Figure 5.14). 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 that coincides with the successful completion of the oviposition event. However, any significant change in the expression of the receptor transcripts in response to second blood meal did not observe (Figure 5.14).

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.

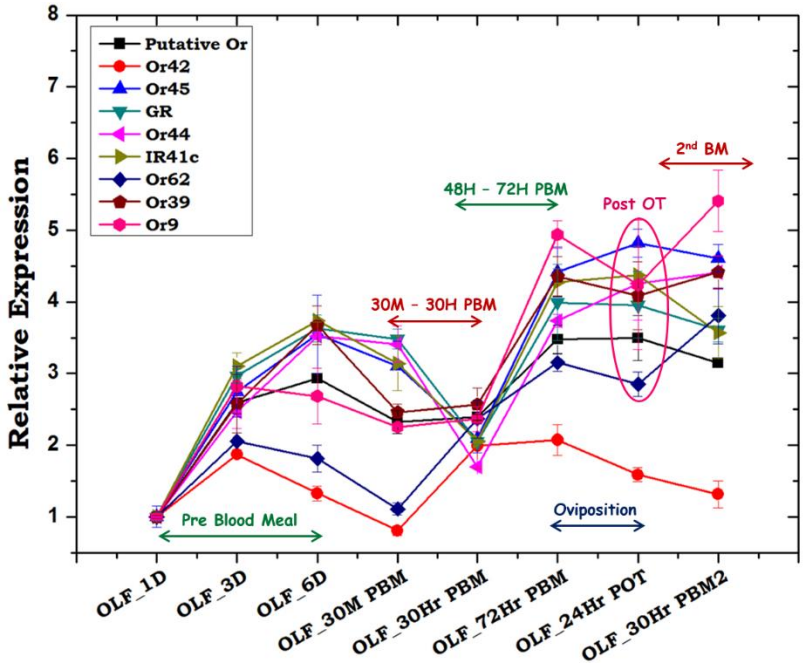

Figure 5.14: Transcriptional response of olfactory receptor genes according to blood meal time series experiment. Olfactory tissues (OLF) were collected from naïve sugar fed adult female mosquitoes till 6th day (OLF-1D, OLF-3D, OLF-6D). Then mosquitoes were provided blood meal and again olfactory tissues 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.

5.3.4 Blood meal response to other olfactory proteins

To test and characterize other chemosensory class of olfactory proteins that were identified from the transcriptomic data, a detailed transcriptional profiling was also performed as described above. 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 (Figure 5.15a). Although, the involvement of G-proteins and related metabotropic signaling mechanism in the olfactory signal transduction of insects remain controversial. But, a rapid and consistent induction of adenylyl cyclase gene after 30m PBM (Figure 5.15b), supported the previous hypothesis that the synthesis of the secondary messenger, cAMP by adenylate cyclase may facilitate odorant mediated signal transduction process which further influence downstream behavioral responses.

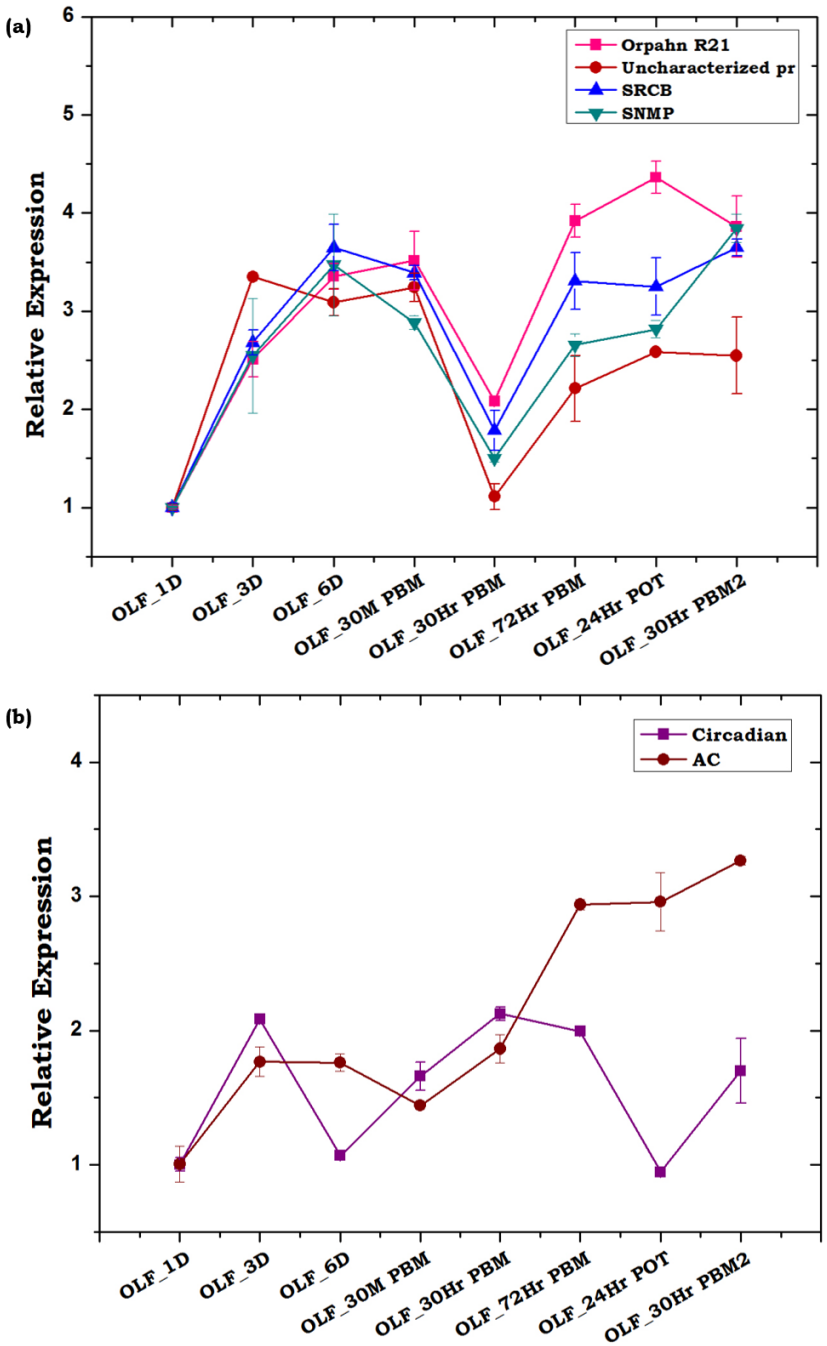

Figure 5.15: Transcriptional responses of other olfactory genes hypothesized to play a crucial role in host-seeking and blood-feeding behavior. (a) Relative expression profiling of other receptor genes according to blood meal time series (described in fig. 5). Orphan R21: Orphan receptor 21; Uncharacterized Pr: Uncharacterized protein; SRCB: Scavenger Receptor class B; SNMP: Sensory neuron membrane protein. (b) Transcriptional profiling of other signaling 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.

5.3.5 Molecular cataloguing of olfactory Immunome

In order to identify the immune genes that are expressed in the olfactory tissue of mosquito, all the transcriptome datasets were compared and screened against the insect ImmunoDB database (http://cegg.unige.ch/Insecta/immunodb). This analysis generated a catalogue of 437 immune transcripts (constituting less than 1% of the total annotated olfactory transcripts) that were classified into 18 immune family proteins (Figure 5.16). Difference in the abundance of transcripts of the member of Clip domain serine proteases (CLIP), Autophagy (APHAG), Peroxidases (PDRX), Scavenger Receptors (SRC), Inhibitor of apoptosis (IAP), Caspases, C-type lectins (CTL) and Polyphenol oxidases (PPO) were observed in the blood fed olfactory tissue sample as compared to naïve mosquito which suggested the maintenance of a basal level of sterility is essential for proper olfactory functions (Figure 5.15).

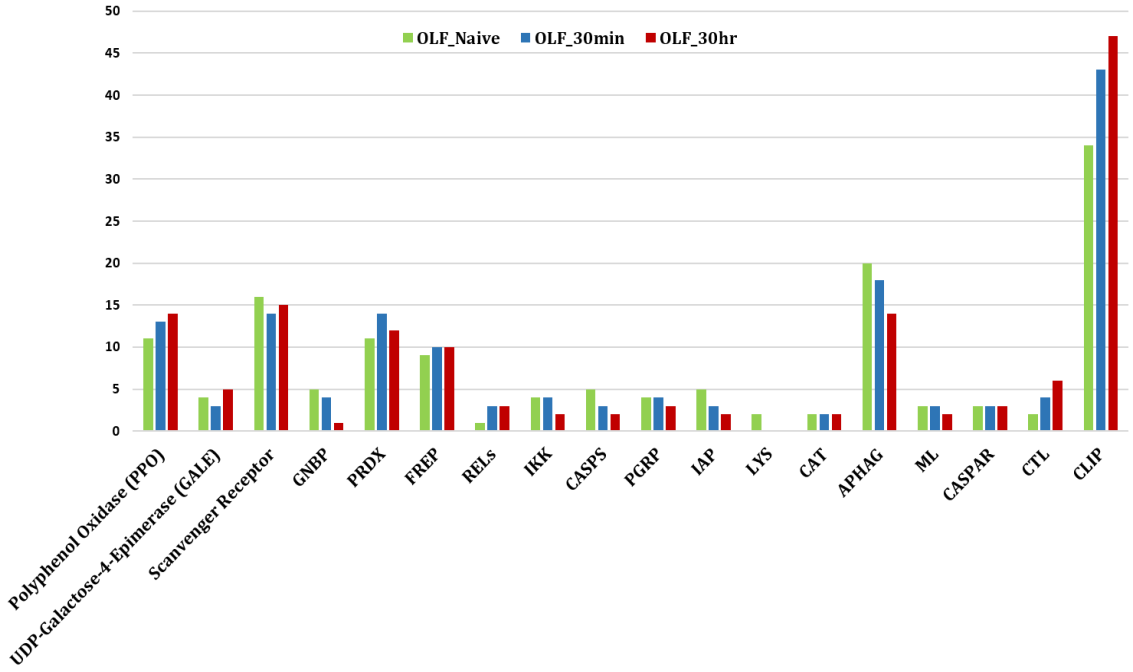

Figure 5.16: Differential expression pattern of olfactory-specific immunome. The immune genes expressed in the olfactory system of *An. culicifacies* are retrieved through BLASTX analysis against the insect ImmunoDB database and categorized into eighteen different family members and their differential expression pattern was determined by the number of sequences appeared in each RNA-Seq data of naïve and blood-fed mosquitoes.

5.3.6 Behavioral difference between *Anopheles culicifacies* and *Anopheles stephensi* in the perspective of olfactory responses

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 [18,145]. In nature, all the members of *An. culicifacies* are predominantly zoophilic, while *An. stephensi* exhibit predominantly anthropophilic behavior [17,18]. 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. Though the molecular basis of such biological variation is yet to unravel, but emerging evidence suggests a significant genetic difference exists among the *Anopheline* mosquito species, including *An. stephensi* and *An. culicifacies* [10,146]. Under laboratory investigation, we frequently observed that biological rhythm may have a significant influence on the biting and blood feeding behavior of *An. culicifacies* than *An. stephensi*. 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 [147–149].

Thus, to test whether any species-specific olfactory derived genetic factors have any differential regulation, influencing the behavioral responses, the expression of at least 6 OBPs transcripts were compared between two laboratory reared mosquito species *An. stephensi* and *An. culicifacies*. In this analysis, two new SAP proteins were also included, which showed a high induction than other OBPs in the olfactory system of the mosquito *An. culicifacies* at midnight (Figure 5.10). 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* (Figure 5.17a) as compared to *An. stephensi* (Figure 5.17b). Together these data indicated that *An. culicifacies* may draw an extra advantage of having more sensitive appendages, possibly to favor more active late night foraging behavior, than *An. stephensi*. Though, defining the basis of host preference remains uncertain, because *Anopheline* mosquitoes have opportunistic feeding behavior which is largely influenced by nature of the host availability [150]. A close association of Ac-SAP proteins with the Anthropophilic *Anopheline* mosquitoes (Figure 5.17c and 5.18a), 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 modeling 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 modeling 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%, favoring their suitability for structure prediction. Out of the 50 modeled 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 (Figure 5.17d and Figure 5.18b).

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 (Figure 5.17e and Figure 5.18c). A best-fit ligand binding site prediction analysis within the selected models was scored by COACH server, based on the consensus, 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 residue 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 (Figure 5.16f and Figure 5.17d). 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 (Figure 5.17g and Figure 5.18e).

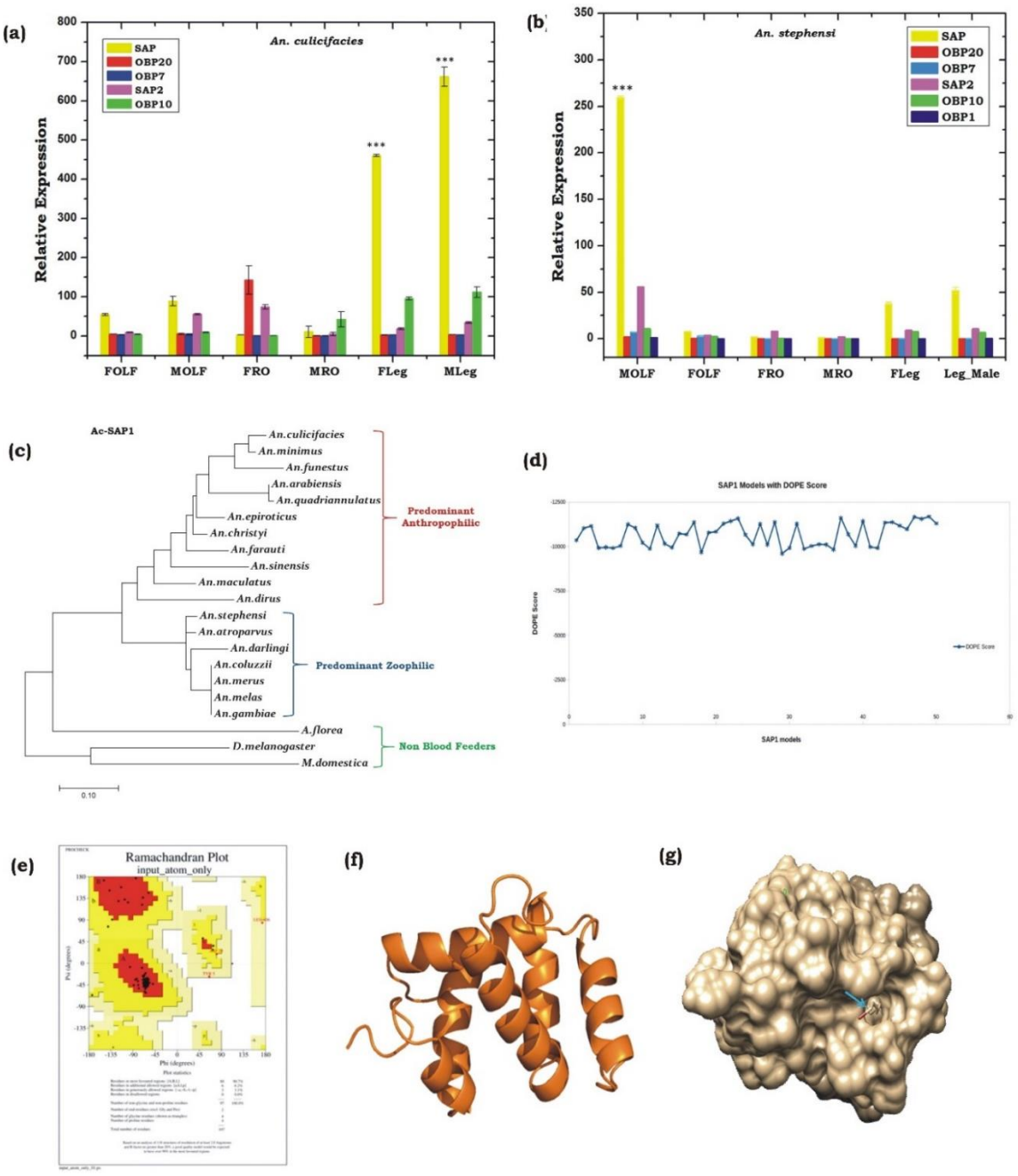

Figure 5.17: Comparative transcriptional responses of Odorant binding protein genes between two major Indian vectors and structural characterization of SAP1. (a, b) Sex and tissue-specific relative expression profiling of OBP genes in *An. culicifacies* (a) and *An. stephensi* (b). FOLF: female olfactory 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. culicifcaies* SAP1 (Ac-SAP1) gene. (d) DOPE score analysis for SAP1. (e) Ramachandran Plot of SAP1 protein. (f) 3-dimensional protein structure of the Ac-SAP1 protein. (g) The binding site of SAP1 protein showed in space fill with nearby residues in stick form.

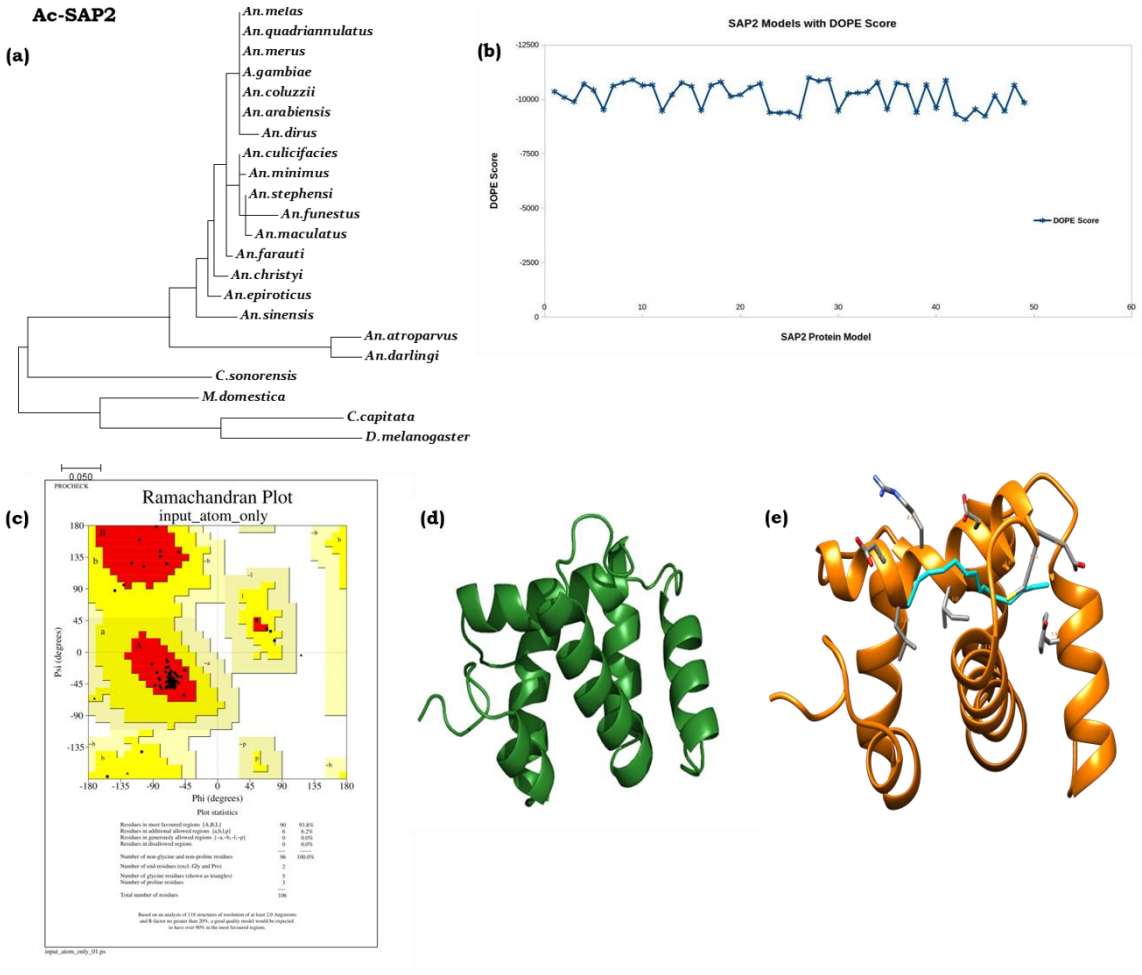

Figure 5.18: Phylogenetic and structural analysis of SAP2. (a) Phylogenetic analysis of *An. culicifcaies* SAP2 gene (Ac-SAP2). (b) DOPE score analysis for SAP2. (c) Ramachandran Plot of SAP2 protein. (d) 3-dimensional protein structure of the Ac-SAP2 protein. (e) The binding site of SAP2 protein showed in space fill with nearby residues in stick form.

5.4 Conclusion

A synergetic action of OBPs/ORs may manage behavioral responses: Adult female mosquito olfactory tissue transcriptomic study coupled with real-time PCR mediated relative gene expression analysis demonstrated that a synergistic and harmonious action of olfactory encoded unique factors may govern the successful 'prior and post' blood feeding associated behavioral complexities. 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 varieties of odorants which are crucial for their every life cycle stages. Furthermore, 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 may facilitate learning for second blood feeding events (Figure 5.19).

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 activities. 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 events.

In summary, we decoded and established a possible functional correlation that how coherent and smart actions of olfactory encoded factors enabled adult female mosquitoes to meet and manage the blood feeding associated complex behavioral activities (Figure 5.19). Furthermore, targeting species-specific unique genes such as sensory appendages proteins may be crucial to design disorientation strategy against the mosquito *An. culicifacies*, an important malaria vector in rural India.

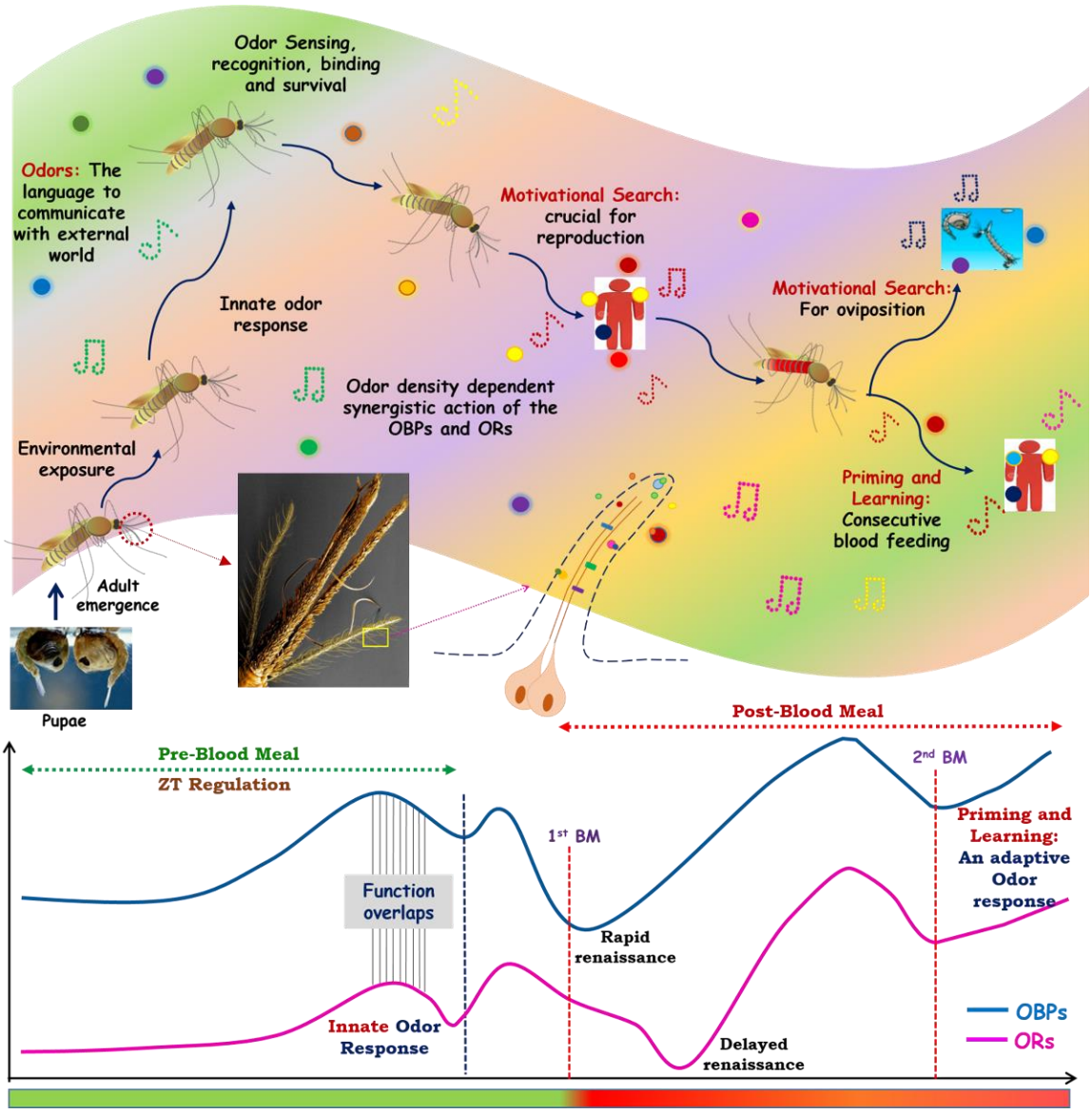

Figure 5.19: How smart actions of olfactory system manages blood feeding associated odor response: an evolutionary specialty of adult female mosquitoes

Chapter 6: Unraveling Molecular Complexity of Mosquito Brain Tissue in Response to Blood Feeding in *Anopheles culicifacies* Mosquito

6.1 Introduction

To guide and manage the diverse nature of external/internal stimuli, the Central Nervous System (CNS), which is also called as the brain, play a central role in shaping every animal's behavior from lower to higher taxa [151,152]. Each and every behavior of any organism is finely orchestrated by multiple organs and brain needs to play a significant role by receiving and processing of the stimuli followed by an exchange of decision making actions and enabling optimal balance and coordination among the internal organs. The common speculation that the larger brain can perform more complex task, may not always be true, at least in case of insect's tiny brain, which can perform amazingly sophisticated behavior [153].

Rapid evolution and adaptation of the insects' taxa reinforce us to hypothesize that insects are either enough smart to squeeze more information from limited number of neurons or they use some different algorithms to process information in their microscopic hardware system to execute complicated output [153]. More surprisingly, it is yet too complex to understand that how the tiny brains of blood feeding mosquitoes manage more challenging diverse functions such as finding a suitable source for sugar feeding and blood feeding, searching for a mate partner and locating a proper oviposition site for egg laying etc. In fact, these behaviors are initiated by the detection of site-specific chemical cues through the olfactory system [91,154,155]. But integration of the initial information with the final decision making events require the retrieval of odor formation from the primary neural code and processing of that information in the miniature brain of mosquitoes. Especially adult female mosquitoes need to manage more diverse and complex behaviors of blood feeding, ovary development and egg laying events, where decision making is very crucial for the fitness of the mosquitoes.

Since adult female mosquitoes have been found to show strong host preference competencies which is also significantly modulated by the aversive/defensive behavior of the available vertebrate host species, therefore, the initial olfactory stimulus may be crucial for the identification and differentiation of individual host species from the pool. But, the molecular and structural basis that how the sex-specific specialization of adult female mosquitoes tiny brain enabling them to learn from the first encounter with a particular

vertebrate host and follow the rewards for the consecutive gonotrophic cycle and thus facilitate their adaptation as fast blood feeders, is not well understood.

Though the current understanding of the mosquito's olfactory system is rapidly expanding, but we do have very limited knowledge that how an adult female mosquito's brain manages pre and post-blood meal associated complex behavioral events. Our observation suggests that a synergistic transcriptional regulation of the olfactory factors is essential to drive blood feeding associated behavioral activities and thus well-coordinated actions of the central nervous system must follow the olfactory instructions. But, once mosquitoes take blood meal brain also needs to deal with high metabolic activities of distant organs, engaged in multiple physiological processes such as, (a) initiation of diuresis to remove the water content of the blood, (b) extension of midgut after blood meal uptake send the signal of satiate state towards the brain to stop mosquitoes to take further blood meal, (c) finding a resting site for blood meal digestion, (d) distribution of amino acids, which have generated through the degradation of protein rich blood meal, for future energy reserves and for egg development etc.

Thus, a large knowledge gap exists that how mosquito's brain (i)coordinate and manages olfactory guided conflicting demand of dual feeding i.e. sugar vs. blood feeding; (ii) synchronize with other tissues viz. midgut, Malpighian tubules, fat body, hemocytes, ovary etc. which undergoes a significant modulation as a result of metabolic switch after blood meal uptake. Though the nature of this neuro-olfactory coordination is not well understood, we hypothesize that brain may undergo dual mode of decision making actions governing olfactory guided pre-blood meal phase and post blood meal inter-organ communication to manage metabolic switch activities (Figure 6.1).

Thus, to decode the possible relationship of neuro-olfactory coordination regulating the multiphasic actions of the brain, we sequenced, cataloged and compared the transcriptional response of the mosquito brain modulating from pre-blood meal to post blood meal physiological status.

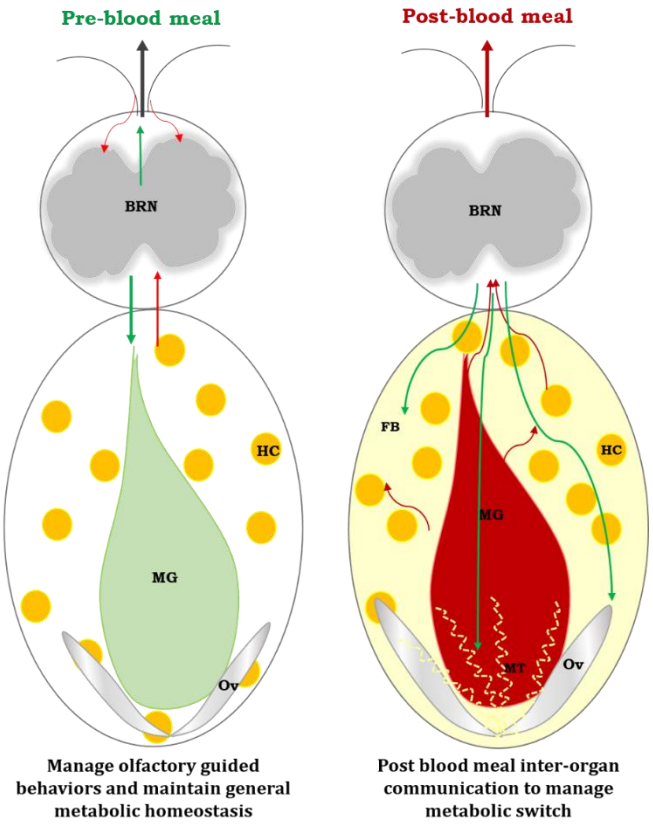

Figure 6.1: A working hypothesis to establish functional co-relation of the brain tissue under distinct feeding status. The brain is the central regulator of every behavior from lower to higher organism. In adult female mosquitoes, sugar fed status of brain manages daily behavioral activities by coordinating with the internal physiological status as well as external stimuli. But, as soon as mosquitoes take blood meal then hyper-activation of brain (BRN) occurs to manage multiple physiological events such as diuresis process mediated by Malpighian tubule (MT) to trace out water content of the blood, then regulating active blood meal digestion in the midgut (MG), then coordinate with MG, Fat body (FB), Hemocyte (HC) and ovary (Ov) for egg maturation and vitellogenesis.

6.2 Material and methods

6.2.1 Tissue collection and molecular analysis

We followed exactly the same protocol for mosquito rearing, tissue collection, RNA-Seq analysis and PCR amplification of the shortlisted genes that are described in chapter 5. For the brain tissue dissection and collection, first cold anesthetized adult female mosquitoes were decapitated and place the heads with the olfactory tissues on a glass dissection slide containing a drop of nuclease-free sterile water. Then, with the help of a fine needle, the olfactory tissues were held stably and gentle pressure was applied over the head to pull out the brain from the head cuticle. Further, any excess surrounding tissues and eye pigment

were removed using the fine needle. Finally, with the help of pipetting tips, the brain tissues were collected in 100 µL of Trizol. For each experiment, ~ 30 adult female mosquitoes' brain were dissected and pooled in a single eppendorf for further molecular analysis. Figure 6.2 represents the complete workflow and technical overview of tissue collection, RNAseq mediated transcriptome data generation and the bioinformatics pipeline that was followed for molecular cataloguing and functional annotation of adult female mosquito's brain tissue.

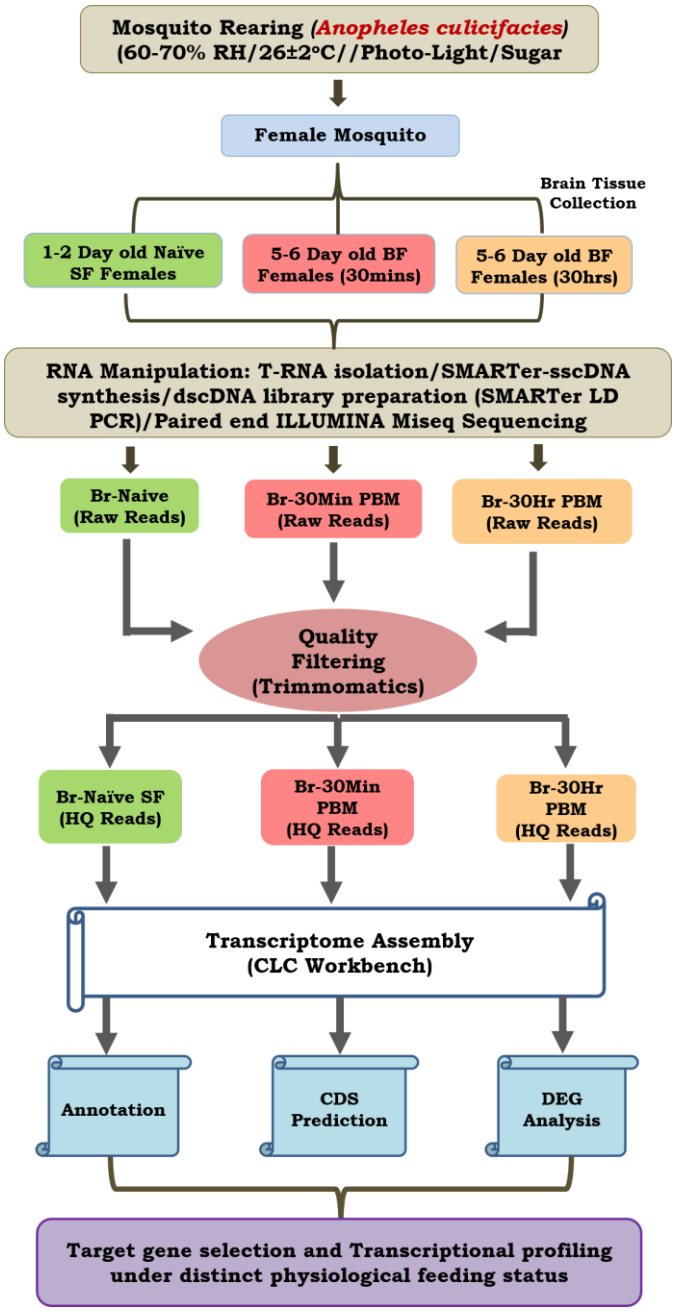

Figure 6.2: A technical overview to decode the hard-wired genetic structure of brain system of *Anopheles culicifacies* mosquito.

6.2.2 Blood meal time series experiment

For detail blood meal time series experiment, the brain tissues were collected from both naïve sugar fed and blood fed mosquitoes at different time points. At first, the brain tissues were collected from 5-6 day old 25-30 naïve sugar-fed adult female mosquitoes. Next, adult female mosquitoes of the same cohort were offered blood meal by offering a live animal (rabbit) and immediately brain tissues were collected for 5 minutes' time point. Later, the full blood-fed mosquitoes were separated and kept in a proper insectary condition and the brain tissues were collected at the following time point of post blood feeding (PBM): 2hr PBM, 10hr PBM, 24hr PBM, 36hr PBM, 48hr PBM, 72hr PBM for detail expression analysis of the respective neuronal genes.

6.2.3 Primer designing

For primer designing, we followed exactly the same protocol as described in chapter 5. Following is the list of primers sequence that was used in the current work (Table 6.1).

Table 6.1: List of primer sequences used in the current study:

Sl No.	Primer Name and Sequence
1.	Slowpoke_Fw: 5'GTTGGGCAGATATCGTTGTA 3' Slowpoke_Rev: 5' GGTTGGCAGTAAGGAAAAGT 3'
2.	cGMP PK_Fw: 5' GCGTTTGATTATCTGCACTC 3' cGMP PK_Rev: 5' AAGGACTCCAAGTGACCAGT 3'
3.	DopR_Fw: 5' GTTATGGGCGTGTTTATTGT 3' DopR_Rev: 5' GCTGGTACTTGCGTCTTATC 3'
4.	Pumilio_Fw: 5'GACAAATTTTCGCTCAAGAC 3' Pumilio_Rev: 5' CCATTGTTTACAGTGGTTCC 3'
5.	5-HT Receptor_Fw: 5' ATGATCTCGCGTAACTCCTC 3' 5-HT Receptor_Rev: 5' ATCGGATTGACCAGACTGC 3'
6.	PI-4Kinase_Fw: 5' ACATCATCTCCTCACTGTCC 3' PI-4Kinase_Rev: 5'GTGTGCCACTGTTGTAATCA 3'
7.	GABA ClCh_Fw: 5' GGAAGGTGTTTGGTAAGTCA 3' GABA ClCh_Rev: 5' GGTGATCGTGTTCGAGTAAT 3'
8.	PLC_Fw: 5' TGGATTCGTCCAACTATCAT 3' PLC_Rev: 5' TTCACGATCACCTCGTTC 3'
9.	GlutamateR_Fw: 5'AGTGGTATCAACGCAGAGTG 3' GlutamateR_Rev: 5' GAGTTTAAGCACTGCTCCAC 3'
10.	NTGated IonCh_Fw: 5' ACGTTTCGAAAGTCAAACAC 3' NTGated IonCh_Rev: 5' GCTGTAGAATGCACAAATGA 3'

11.	Glycine R_Fw: 5’ GATACTGCCACTACCTCGTC 3’ Glycine R_Rev: 5’ CTTGGAGACCGAATTGAATC 3’
12.	GABA R_Fw: 5’ CAGAACGAAGAAGGCTACTC 3’ GABA R_Fw: 5’ AGTATCCACGCATACTCAGC 3’
13.	ST ProteinKinase_Fw: 5’TTTATAGTGCCGTGTGTTGA 3’ ST ProteinKinase_Rev: 5’CTTAATGTGGAACCGATCAT 3’
14.	Octopamine Receptor_Fw: 5’CTACTGGCGGATCTATCGGG 3’ Octopamine Receptor_Rev: 5’ TGGTGGAAGGCTGTGTTTTG 3’

6.3 Results and discussion

6.3.1 Analysis of brain transcriptome of *An. culicifacies* mosquito

To decode the detail molecular architecture of the brain modulating in response to blood meal we analyzed a total of ~129 million RNA-Seq reads originating from the brain tissues collected from 1-2 day old naive (Nv), 5-6-day old immediate blood fed (30m-2h PBM) and 30hr post blood fed (30hr PBM) mosquitoes. A *denovo* clustering of the raw reads was performed using CLC Genomics Workbench (V6.2) to build final contigs/transcripts dataset. For comprehensive molecular and functional annotation analysis, we compared the assembled transcripts of each set against multiple molecular databases such as non-redundant, GO, PDB, KEGG etc. Table 6.2 represents the annotation kinetics of brain transcriptome data.

Table 6.2: Annotation kinetics of RNA-Seq data

Molecular Features	**Ac-OLF-Naive**	**Ac-OLF-30M PBM**	**Ac-OLF-30Hr PBM**
Total No. of Raw Reads	5268211	3947521	3760078
Total No. of Contigs	32118	32984	38512
Total Transcripts	9460	9146	7387
Total BLASTx hits (NR)	8,668 (~91%)	8,336 (~91%)	6,548 (~88%)
Transcripts with GO Match			
Molecular Function	4773	4556	3575
Biological process	4446	4299	3381
Cellular component	2523	2424	1888

6.3.2 Blood meal-induced gradual alteration of transcripts abundance may influence brain responses

To test whether blood meal alters the global expression pattern of the brain transcripts, we performed a global gene expression analysis. Our initial read density map analysis suggested that blood meal may cause a gradual change in brain transcripts expression pattern (Figure 6.3). A detail differential gene expression analysis further revealed that more than 80% brain transcriptome remains unaltered in response to first blood meal, however, a unique observation of a time-dependent gradual alteration of transcripts abundance i.e. a down-regulation of at least ~11% transcripts within 30 min, followed by further depletion to ~14% transcripts (Table 6.3), suggested that blood meal may have a significant influences on the brain function to manage blood meal specific responses.

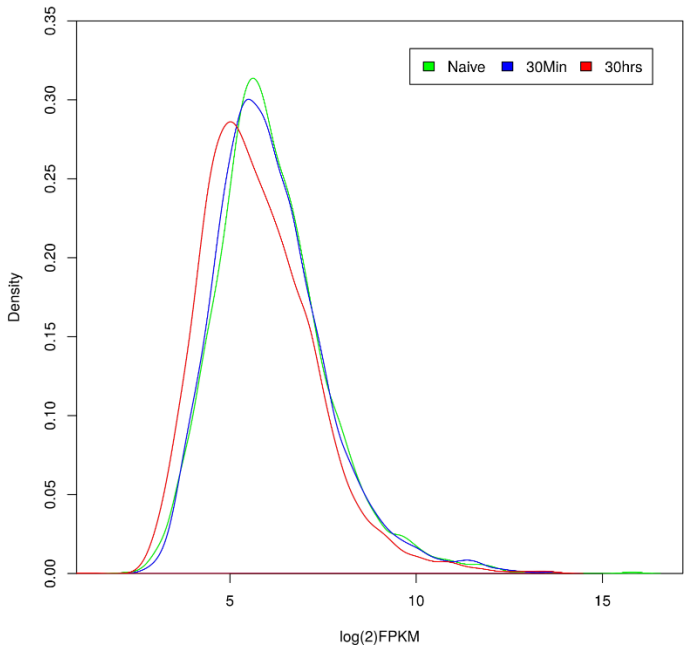

Figure 6.3: Blood meal causes modest change in the molecular architecture of the mosquito brain system. Read density map of the compared Naive; 30M and 30Hr post blood meal (PBM) transcriptomic data of brain tissue.

Table 6.3: Percentage of differentially expressed brain transcripts

Sample	No. of Transcripts	Transcripts showing Differential gene Expression (DGE)	Upregulated Transcripts	Downregulated Transcripts	Percentage of Transcripts showing DGE
Ac_Br_naive vs Ac_Br_30min	(9460 + 9146) = 18606	Total - 4747 Significant – 3183 Not significant - 1564	622 (3%)	2110 (11%)	14% CDS show differential expression
Ac_Br_Naive vs Ac_Br_30hr	(9460 + 7387) = 16847	Total -3966 Significant – 3174 Not significant - 792	482 (2%)	2469 (14%)	16 % show differential expression

Comprehensive differential gene expression analysis of common transcripts revealed that compared to naïve mosquito brain, at least 39 transcripts showed more than 5 fold up or down-regulation, highlighting the significant modulation of brain function in response to blood meal. Notably, up-regulation of juvenile hormone binding protein after blood feeding supported the previous report that JH plays a central role in the regulation of yolk protein synthesis and facilitate egg maturation [115,121]. The high abundance of oxidative stress response gene and DNA repair genes in the blood fed brain possibly may manage brain health from the potent toxic effect of oxidative change occurring as a result of blood feeding (Table 6.4a). A significant alteration of distinct signaling molecules and transcription regulator genes further indicated that the brain may undergoes a hyper-active status to meet and manage blood meal associated complex metabolic and physiological changes (Table 6.4a, b).

Table 6.4a: List of upregulated genes in brain tissue in response to blood meal

Sl. No.	Accession	Origin of the Transcript	Log Fold Change	Putative Function
1.	XP_564139	Br_30min_CDS_6018	7.249014 up regulated	Break down of glycosaminoglycans
2.	XP_310955	Br_30min_CDS_338	6.981352 up regulated	Purine biosynthesis

3.	XP_317285	Br_30min_CDS_3994	6.703983 up regulated	Haemolymph juvenile hormone binding protein
4.	XP_321213	Br_30min_CDS_5612	6.68632 up regulated	Oxidative stress response/DNA repair
5.	XP_003436018	Br_30min_CDS_8107	6.390301 upregulated	Hypothetical protein
6.	XP_311158	Br_30min_CDS_7653	5.853747 up regulated	Transcriptional activator
7.	AAO06829	Br_30min_CDS_3628	5.409987 up regulated	Apyrase
8.	XP_310923	Br_30min_CDS_4612	5.3684 up regulated	Lipase
9.	XP_312566	Br_30min_CDS_5463	5.180243 up regulated	DNA Repair
10.	XP_316296	Br_30min_CDS_5812	5.06028 up regulated	Esterase
11.	XP_001656922	Br_30h_CDS_2504	9.758 up regulated	Protein glycosylation
12.	XP_310382	Br_30h_CDS_7125	9.362 up regulated	Hypothetical Protein
13.	XP_001688203	Br_30h_CDS_4220	9.338 up regulated	Phenylpropanoid metabolic process
14.	XP_313831	Br_30h_CDS_306	8.208 up regulated	hydroxymethylglutaryl-CoA synthase
15.	XP_315872	Br_30h_CDS_3931	8.116 up regulated	Protein Phosphase/Signaling
16.	XP_313783	Br_30h_CDS_3530	7.3532 up regulated	Actin filament binding
17.	XP_001688022	Br_30h_CDS_5825	6.7120 up regulated	DNA repair
18.	XP_311530	Br_30h_CDS_6124	6.3492 up regulated	tRNA-specific adenosine deaminase/RNA editing
19.	XP_553120	Br_30h_CDS_5108	5.6988 up regulated	rRNA processing
20.	XP_312240	Br_30h_CDS_3880	5.5575 up regulated	rRNA processing
21.	XP_309424	Br_30h_CDS_6760	5.4817 up regulated	Cell division

Table 6.4b: List of downregulated genes in brain tissue in response to blood mea

Sl. No.	Accession	Origin of the Transcript	Log Fold Change	Putative Function
1.	XP_317561	Br_30min_CDS_7560	-11.5314 downregulation	glucosyl/glucuronosyl transferase
2.	XP_311155	Br_30min_CDS_4005	-10.9044 downregulation	High mobility group protein DSP1/DNA binding
3.	XP_001237211	Br_30min_CDS_5360	-9.82137 downregulation	zinc finger protein/ Transcriptional Regulation
4.	XP_321321	Br_30min_CDS_5258	-9.71664 downregulation	Ceramide synthesis
5.	XP_312081	Br_30min_CDS_6201	-9.59305 downregulation	Protein tyrosine phosphatase/Signaling
6.	XP_309868	Br_30min_CDS_2971	-9.55864 downregulation	Happyhour
7.	CAD59403	Br_30min_CDS_5051	-6.2986 downregulation	Structural maintenance of chromosomes protein 1
8.	XP_310896	Br_30min_CDS_6588	-5.76943 downregulation	serine/threonine-protein kinase
9.	XP_313558	Br_30min_CDS_2438	-5.55182 downregulation	SET and TTL domain-containing protein/Transcriptional regulator
10.	XP_312887	Br_30min_CDS_6686	-5.40114 downregulation	DNA mismatch repair protein mut
11.	XP_311155	Br_30hr_CDS_4175	-11.2785 down regulated	High mobility group protein DSP1/DNA binding
12.	XP_001237211	Br_30hr_CDS_7188	-11.0417 down regulated	Uncharacterized
13.	XP_312081	Br_30hr_CDS_4875	-8.16956 down regulated	Protein tyrosine phosphatase 99A/ axonal defasciculation
14.	XP_317275	Br_30hr_CDS_5988	-5.94007 down regulated	Nidogen/Cell adhesion
15.	XP_313558	Br_30hr_CDS_6300	-5.60451 down regulated	SET and TTL domain-containing protein/Transcriptional regulator
16.	XP_316184	Br_30hr_CDS_5076	-5.3791 down regulated	tRNA methyltransferase
17.	XP_312887	Br_30hr_CDS_6804	-5.364 down regulated	DNA mismatch repair protein mut
18.	BAC57908	Br_30hr_CDS_3536	-5.24237 down regulated	Reverse Transcriptase

6.3.3 Blood meal-induced oxidoreductase family proteins may manage metabolic switch: A comprehensive functional annotation and a comparative GO score distribution analysis also indicated a gradual decrease in the brain responses linked to distinct biological process (Figure 6.4). However, unexpectedly we also observed an exclusive induction of the transcripts related to oxidation-reduction process in response to blood meal. Together, these data allowed us to hypothesize that after blood meal uptake (i) a gradual alteration of the brain transcripts may enable mosquitoes to temporarily pause the olfactory guided actions and (ii) initiate specialized neuro actions to manage metabolic switch and nutritional homeostasis, possibly through induction of oxidoreductase transcripts.

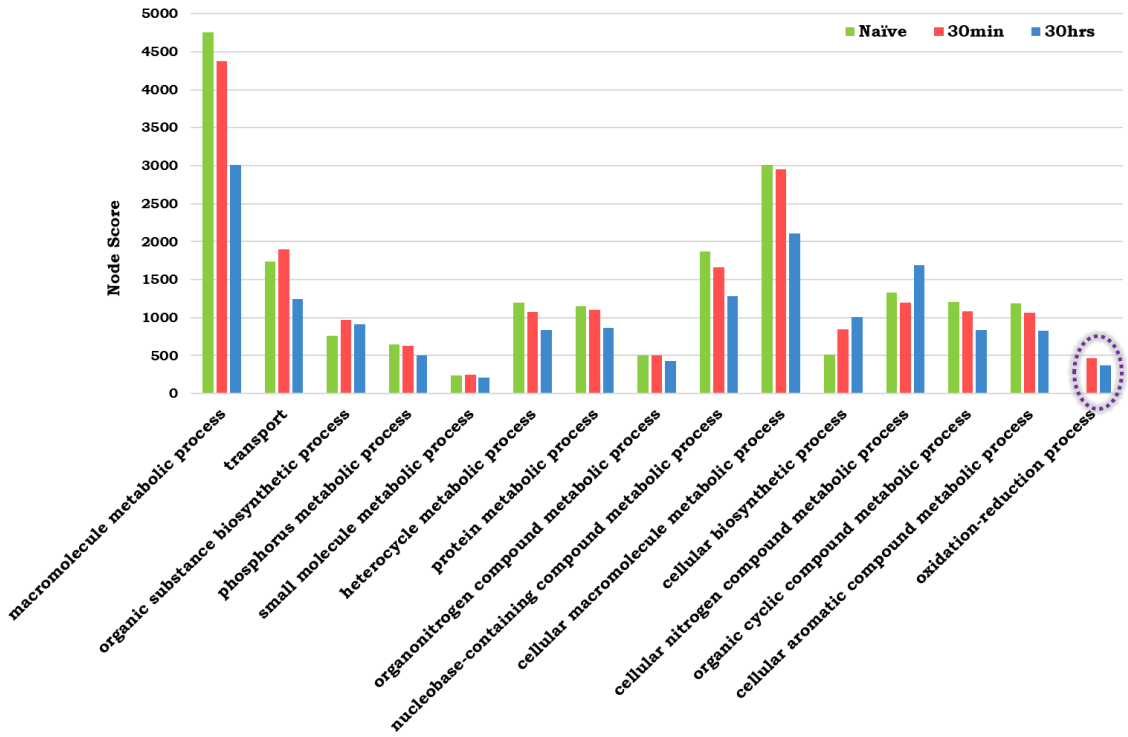

Figure 6.4: Functional annotation and molecular catalogue of brain transcriptome (Biological Process/Level4/Node score). Purple circle highlighted the unique genes that appeared in the brain tissue after blood meal intake.

Immediately after blood meal uptake, mosquitoes need to manage complex events of blood meal digestion and egg maturation, a process which entails multi-organ coordination. Thus, to test whether blood meal-induced oxidoreductase gene expression follows a time-dependent modulation of unique transcripts; we retrieved and compared the molecular and functional nature of the oxidoreductase transcripts. Our initial Venn diagram analysis identified that out of total 441 transcripts, only 223 (54.3%) appears in common, while 119 (29%) and 69 (16.8%) transcripts remain uniquely associated with 30 min and 30hrs (Figure 6.5a) blood-fed mosquito brain, respectively. Furthermore, GO annotation and

comparison analysis unraveled that majority of the transcripts having similar functions, except the genes encoding Regulation of cellular process (3%) and Response to chemicals (3%), which are restricted to 30hrs post blood-fed mosquitoes brain (Figure 6.5b, c). Together, these data supported the hypothesis that a time-dependent changes in the oxidation-reduction associated gene cluster may help the brain to overcome the blood meal-induced stress responses and hence optimally regulate the events corresponding to metabolic switch activities.

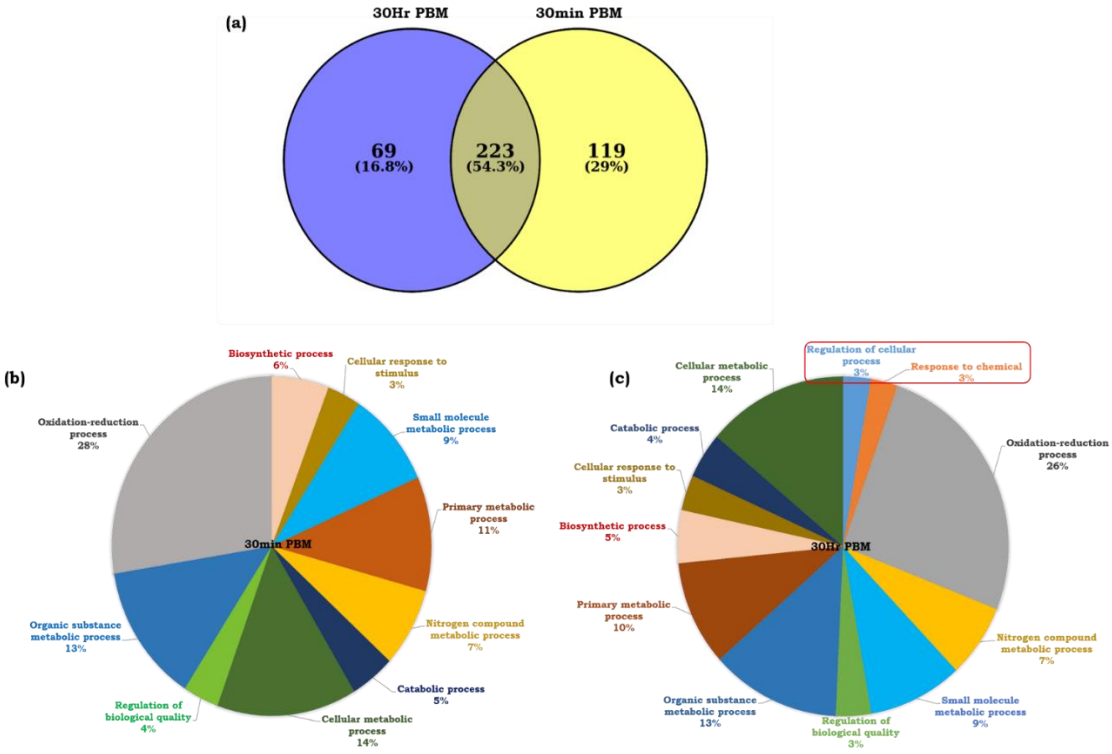

Figure 6.5: Detail cataloguing of oxidoreductase category of genes. (a) Venn diagram showing common and unique transcripts of the oxidoreductase GO category retrieved from brain transcriptome data of 30min and 30hr post blood-fed mosquitoes. (b) A comparative GO score distribution analysis of oxidoreductase transcripts of 30min post blood-fed mosquitoes. (c) A comparative GO score distribution analysis of oxidoreductase transcripts of 30hr post blood-fed mosquitoes.

6.3.4 Blood meal causes hyper activeness of brain function

Though our finding indicated that after emergence from pupae, the active olfactory system of the mosquitoes enables them to derive complex feeding behavioral responses, but it is not known that how brain coordinate with these events. We hypothesize that the naïve mosquito's brain must follow tightly controlled activities to manage decision making

events necessary for their fitness. In the lack of available scientific knowledge, we further opined that post blood meal mosquitoes may be physically inactive, but a great deal of biochemical changes occurring in responses to the metabolic switch which needs to be tightly regulated by the active central nervous system/brain in the adult female mosquitoes.

The activity of the brain can be measured by the frequency of nerve firing which depends on the recurrence of neuronal signal transmission process [156,157]. The key event of neuronal signal transmission process is the synaptic transmission, during which presynaptic neurons release neurotransmitters to induce postsynaptic neuron [157]. In case of the blood feeding mosquitoes, we propose that in the tiny brain the synaptic transmission may follow two signaling phases i.e. (i) receptor-mediated neuronal signaling and (ii) cellular signaling (Figure 6.6). To decode and identify the molecular factors that play a critical role in synaptic transmission, first, we shortlisted and analyzed a few brain-specific transcripts, either based on their expression pattern and/or localization.

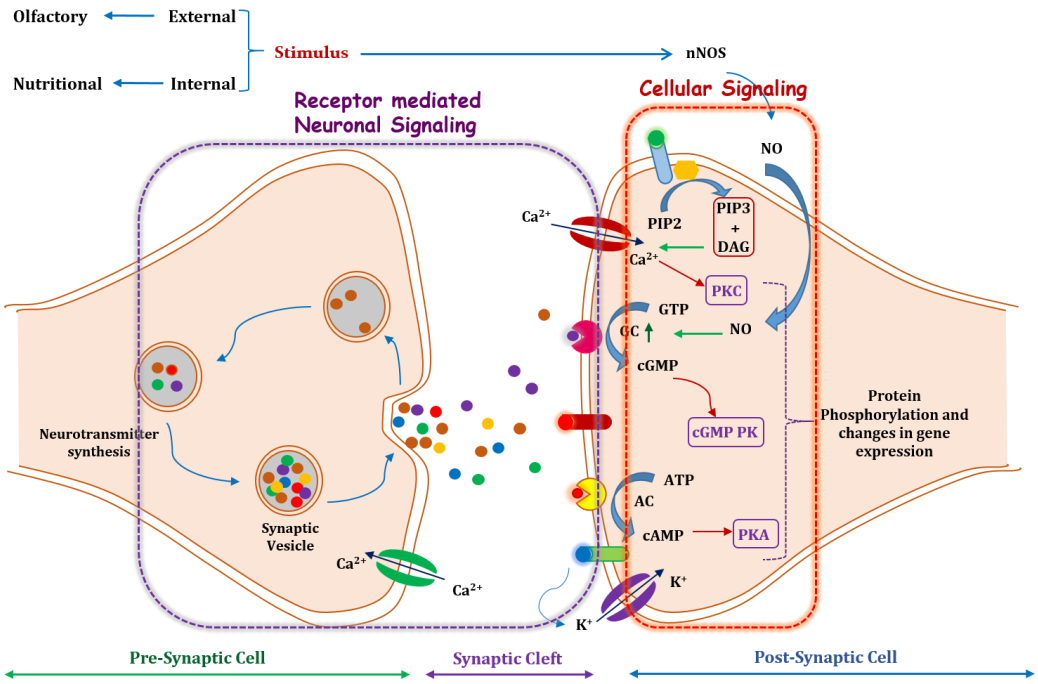

Figure 6.6: Synaptic signal transmission and probable mechanism of neuro-signaling. After activation by external and/or internal stimulus the synaptic vesicle of the presynaptic neuron, containing the neurotransmitters are released into the synaptic cleft. Binding of the respective neurotransmitter with their cognate receptors activate the downstream signal transduction process in the postsynaptic neuron and thus activate and transmit the initial signal through the interconnecting neurons.

To unravel the activity pattern of the brain in the perspective of receptor-mediated synaptic signaling and activation of post-synaptic neuron through cellular signaling mechanism, we monitored and compared the transcriptional responses of the selected transcripts in naïve sugar fed and blood fed mosquitoes. Our relative gene expression analysis of the neurotransmitter and biogenic amine receptor genes such as serotonin receptor, dopamine receptor, octopamine receptor, GABA receptor etc. showed a limited change in response to blood meal, a possible mechanism to prevent the over-excitation of the neurons (Figure 6.7a). While the molecular factors viz. cGMP protein kinase, phospholipase C, GABA gated chloride channel, serine threonine protein kinase etc. which are involved in cellular signal transduction, exhibited a significant modulation in response to metabolic switch (Figure 6.7b). These findings strongly suggested that blood meal uptake may cause an acute change in the metabolic activity inducing physiological alterations in multiple-organs, of the blood-fed mosquitoes. We concluded that blood meal uptake induces a hyperactivity of the brain at least for 24hrs, possibly to regulate blood meal associated complex events ongoing in the distant organs.

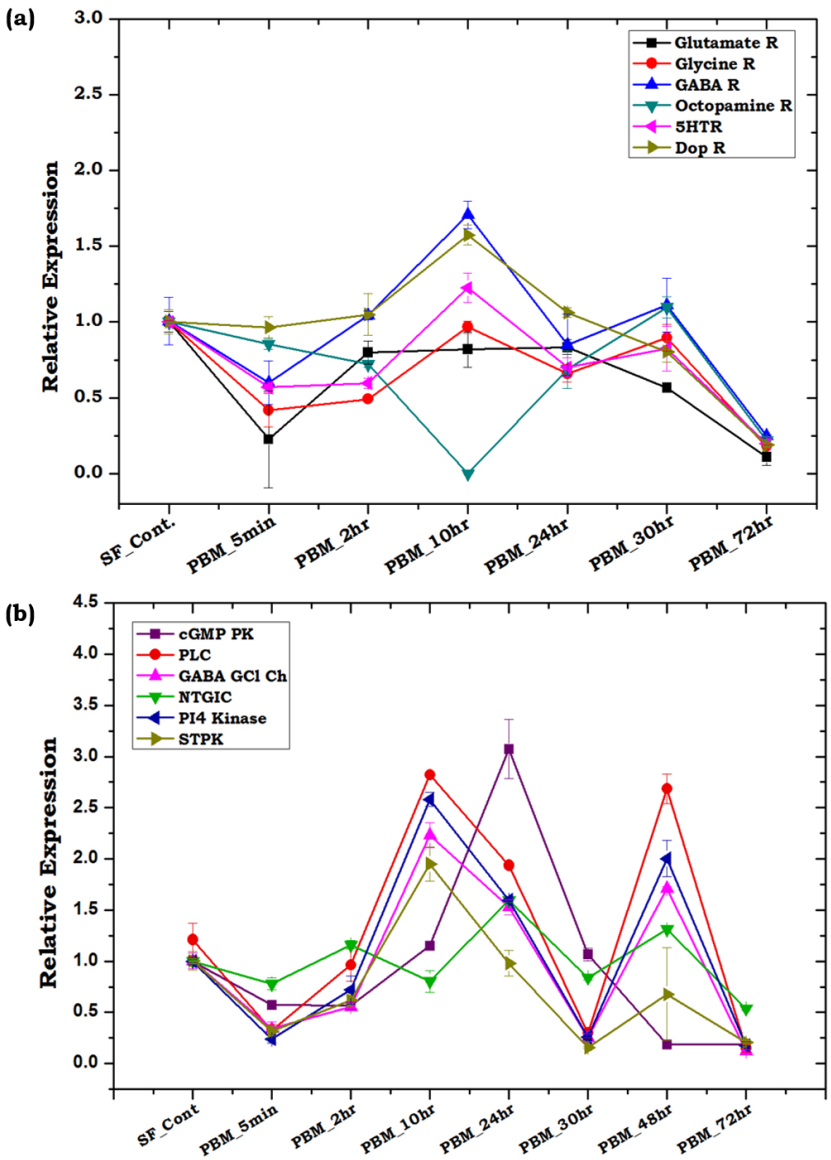

Figure 6.7: Innate physiological status modulates brain transcripts expression. (a) Transcriptional response of neurotransmitter receptor genes according to the detail blood meal time series experiment. Brain tissues were collected from 5-6day old naïve sugar fed adult female mosquitoes. Then mosquitoes were provided blood meal and the brain tissues were collected at different time point after blood feeding viz. 5min post blood meal (PBM-5min), PBM-2hr, PBM-10hr, PBM-24hr, PBM 30hr and PBM-72hr. Glutamate R: Glutamate Receptor; NTGIC: Neurotransmitter Gated Ion Channel; Glycine R: Glycine Receptor; GABA R: Gamma-Aminobutyric Acid Receptor; Octopamine R: Octopamine Receptor; 5HTR: Serotonin Receptor; Dop R: Dopamine Receptor. **(b)** Relative expression profiling of the genes involved in signal transduction molecules according to the detail blood meal time series experiment. Brain tissues were collected from 5-6day old naïve sugar fed adult female mosquitoes. Then mosquitoes were provided blood meal and the brain tissues were collected at different time point after blood feeding viz. 5min post blood meal (PBM-5min), PBM-2hr, PBM-10hr, PBM-24hr, PBM 30hr, PBM-30hr and PBM-72hr. cGMP PK: Cyclic GMP Protein Kinase; PLC: Phospholipase C; GABA GCl Ch: GABA Gated Chloride Channel; NTGIC: Neurotransmitter Gated Ion Channel; PI4 Kinase: Phosphatidyl-inositol-4-Kinase; STPK: Serine Threonine Protein Kinase.

6.3.5 Rhythmic expression of brain transcripts may regulate sleep and wakefulness behavior in mosquitoes

Sleep is a universal event that is tightly regulated by circadian as well as the homeostatic process in both vertebrates and invertebrates. But, the requirement of sleep is an unresolved mystery in any animals. The molecular basis of sleep and wakefulness is partly defined in the model organism *Drosophila sp.* [158,159], where decades of research in the circadian rhythm showed that they are more active during daytime and much less during the night [159].

However, unlike *Drosophila*, different species of mosquitoes display great variability's in their activity and sleep pattern, which may also affect their disease transmission ability [17,135,160,161]. *Anopheles* mosquitoes are predominantly active during night hours to perform the foraging and host-seeking behavior safely, by minimizing the possibility of any host interruption. Our initial study on the olfactory system of *An. culicifacies* mosquito showed that this mosquito species has a midnight biting preference. Thus, further to corroborate brain responses with olfactory actions we monitored the expression pattern of the selected brain transcripts according to the circadian rhythm.

A zeitgeber time scale expression analysis revealed that Dopamine receptor gene and cyclic GMP protein kinase showed a significant modulation, whereas octopamine receptor and serotonin receptor showed a limited change (Figure 6.8). More than 5-fold increase of the dopamine receptor genes during the day time not only support the previous reports [162–165] but also clearly suggested that *Anopheles* mosquitoes are less active during the day. While a sharp down-regulation of this gene at midnight further highlighted the active status of mosquitoes. Simultaneously, the upregulation of cyclic GMP protein kinase after day time also indicated the active neuronal signal transmission during the night when mosquitoes are awake and perform their foraging behavior (Figure 6.8). Together these data indicated that dopamine signaling plays a crucial role in the determination of sleep and wakefulness in the mosquitoes also.

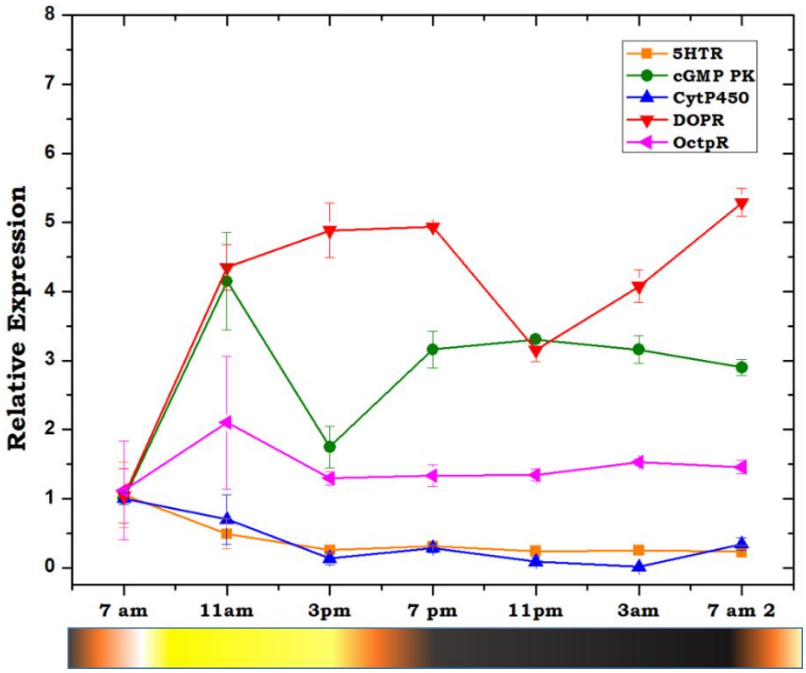

Figure 6.8: Rhythmic expression of brain transcripts according to day-night cycle. 7 am indicates the end of dawn transition, 6 pm indicates the end of dusk transition and initiation of darkness, 12 pm indicates the midnight phase.

6.3.6 Blood meal response to brain immune gene expression

The central nervous system and the immune system are the most energy consuming organs that have a discrete function in any organism. The immune system plays a crucial role in maintaining brain health by protecting it from both external and internal stress [166–168]. Blood meal uptake induces enormous internal stress to the mosquito such as oxidative stress, osmotic stress, elevated levels of dietary heme molecules etc. Thus, we hypothesize that the immune system may play a crucial role to overcome the metabolic stress and keep the brain in shape.

In order to identify the putative immune genes expressing in the central nervous system, we merged the brain transcriptome data of naïve and blood-fed mosquitoes and performed tBLASTX analysis against the insect ImmunoDB database with a cut-off E-value of $\leq 10^{-10}$. Our analysis identified a total of 913 immune transcripts from brain tissue transcriptome data which can be classified into 18 different immune families (Figure 6.9a). Among all the categories autophagy, CLIP-domain serine proteases and peroxidases were observed the most predominant accounting more than 50% of the total immune transcripts. Furthermore, a comparative transcript abundance analysis showed that blood meal may cause a moderate change in the immune transcript expression (Figure 6.9b). Slight up-regulation of peroxidases and CLIP-domain serine protease in blood-fed mosquito brain suggested that these immune transcripts may prevent brain tissue from oxidative stress-

induced damage and facilitate its recovery (Figure 6.9b). Further, functional analysis of the immune transcripts in the central nervous system may unravel the novel regulatory mechanism of the immune genes to maintain the brain in shape.

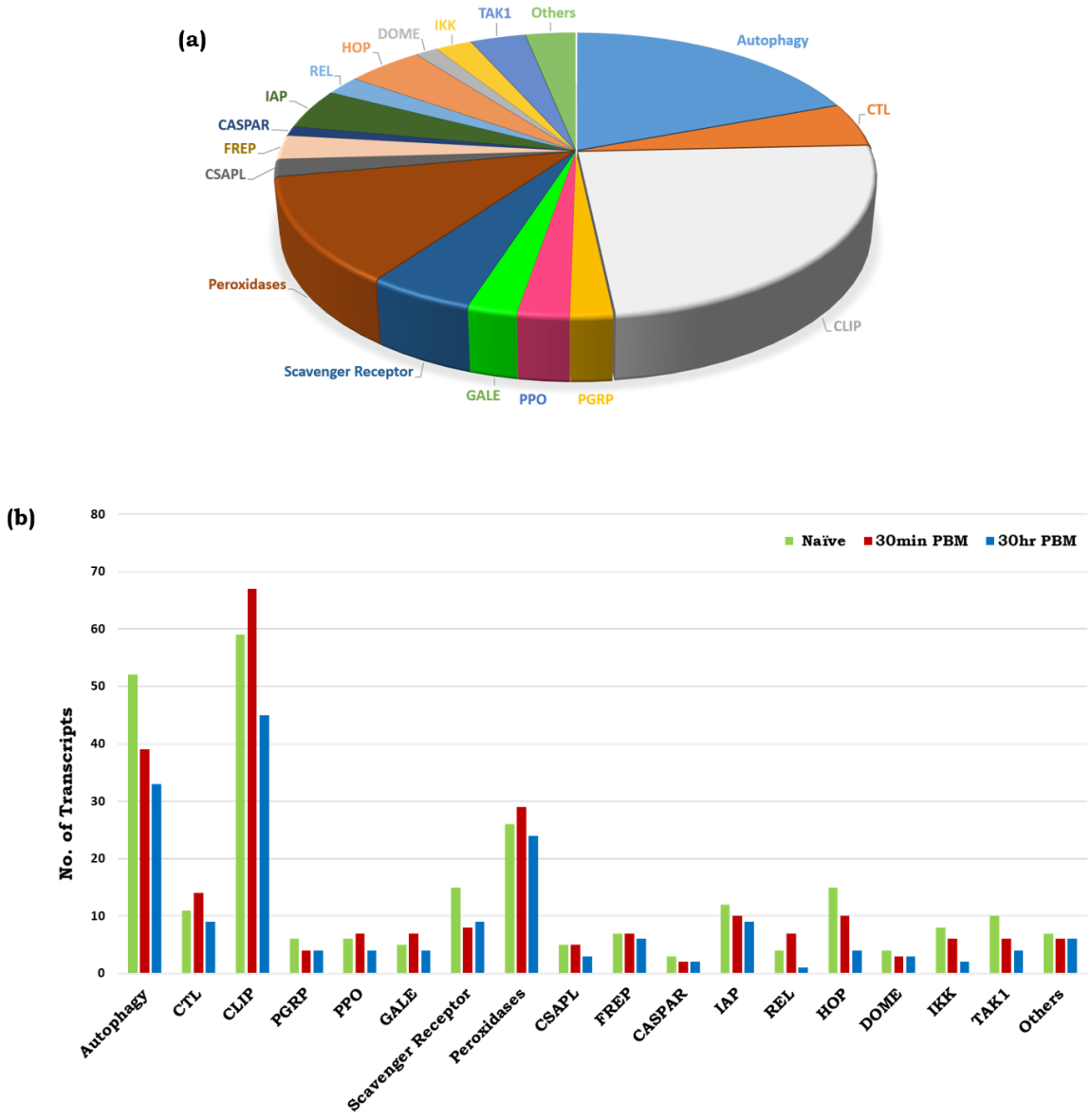

Figure 6.9: Catalogue of brain-specific immunome. (a) Molecular catalogue of the different class of immunity genes expressed in the brain tissue. **(b)** Differential expression pattern of the brain immunome as determined by the number of sequences appeared in each RNA-Seq data of naïve and blood-fed mosquito brain.

6.4 Conclusion

Decoding the genetic relationship that how central nervous system regulates pre and post blood feeding events, may provide a unique opportunity to design new molecular tool to interrupt human-mosquito bite exposure. Our comparative RNAseq analysis of naïve and blood fed adult female mosquitoes unraveled that a gradual modulation of brain transcripts expression is crucial to manage blood meal associated decision-making events. Although, blood meal causes a limited change in the neurotransmitter receptor gene expression, but a significant modulation of GABA receptor, dopamine receptor and a continuous modulation of the transcripts engaged in cellular signaling indicated that GABA and dopaminergic signaling mechanism may be important to regulate the metabolic switch events. Taken together, our findings strongly suggested that despite of the mosquito's physical inactiveness after blood meal intake, their brain is actively engaged to manage the multi-physiological events of distant organs and hence valuable to maintain of the nutritional homeostasis. Unexpected observation of large pool of immune transcripts in the brain tissue highlighted that maintaining an active blood-brain relation is beneficial for brain health and the immune transcripts may protect the brain cells from stress-induced degradation. Future characterization of other brain-specific genes may facilitate the detailed understanding of the neuronal regulation of mosquito feeding behavior

Chapter 7: Molecular and Functional Characterization of the Novel *quick-to-court* Gene (*Ac-qtc*) in the Mosquito *Anopheles culicifacies*

7.1 Introduction

Seasonal elevation of the mosquito population and acute transmission of malaria, dengue etc. has a critical impact by raising the mortality rate and the economic burden. Multiple non-genetic factors such as humidity, temperature, water source richness and rapid growth of vegetation may have a significant impact on this relationship. But how these factors influence the sex-specific behavioral biology, especially feeding, mating and breeding of mosquitoes are yet to be resolved. Compared to the vast research concentrated on female mosquitoes, male mosquito biology is rather less explored possibly due to its indirect influence on parasite transmission. Though, males induce several post-mating behavioral changes in females, which also includes the blood feeding behavior. Thus, the male mosquitoes maintain the continuity of the mosquito life cycle and significantly contribute to diseases transmission in an indirect way. But resolving the process through which mosquitoes manage complex mating behavioral events i.e. swarm formation, suitable mate finding and successful aerial coupling is yet a major challenge to entomologists.

During the course of olfactory tissue transcriptomic data annotation of blood-fed adult female *An. culicifacies* mosquito, unexpectedly, we identified a 383 bp long unique transcript encoding *Drosophila* homolog of coiled-coil Quick-To-Court (*Dm-qtc*) protein. In *Drosophila melanogaster,* it is predominantly expressed in olfactory organs, central nervous system and male reproductive tract [169,170]. Previous studies showed that mutations in the *Dm-qtc* not only causes the males to show an elevated levels of male-male courtship but also favors abnormally quick courtship when placed in the presence of a virgin female [169]. A ~5 fold up-regulation of this unique transcript *Ac-qtc* in response to blood feeding, prompted us to investigate its possible role in managing sex-specific conflicting demands of 'mate choice' and/or 'food choice' in the mosquito *An. culicifacies*. Unlike *Drosophila*, unavailability of the proper molecular marker restricted our understanding of the complex mating biology in mosquitoes.(Mahmood and Reisen, 1994). Thus, we attempted to test whether *Ac-qtc* plays a key role to drive sex-specific behavioral modulation in *An. culicifacies* mosquito. Our initial *in silico* function prediction analysis

and extensive transcriptional profiling established a possible correlation that *Ac-qtc* might play a crucial role in the regulation of mating as well as blood-feeding behavior in the mosquito *An. culicifacies*.

7.2 Materials & Methods

7.2.1 Mosquito rearing and molecular analysis

The detailed protocol of mosquito rearing, maintenance was previously mentioned in chapter 5. Likewise, for tissue collection, RNA extraction, and relative gene expression analysis the same protocol was followed as mentioned in the previous chapter (Chapter 5). Briefly, from the ice anesthetized adult *An. culicifacies* mosquitoes, the desired tissues *viz.* olfactory tissues (that include antennae, maxillary palp, proboscis, and labium), brain, reproductive tissues (male reproductive organ includes testes and male accessory gland; female reproductive organ consists of spermathecae and autrium) and legs of both male and female mosquitoes were dissected and collected in trizol. The developmental stages of *An. culicifacies* viz. egg, larvae (stages I – IV) and pupae were collected in trizol after removal of extra water through filter paper. Total RNA was isolated by the standard trizol method. Figure 7.1 represents a technical overview and workflow of the experiments to demonstrate the possible sex-specific role of *Ac-qtc* in adult *An. culicifacies.*

7.2.2 Multiple sequence alignment and phylogenetic analysis

Following primary BLASTX analysis, the top hits reference sequences were retrieved and edited for subsequent analysis in the FASTA format. Multiple sequence alignment was performed using ClustalX2 software [172,173]. The CLC Sequence viewer (http://www.clcbio.com) software was used for better quality graphics. The phylogenetic analysis of *Ac-qtc* was performed through MEGA7 **(**http://www.megasoftware.net/) software. The evolutionary relationship was retrieved using the Neighbor-Joining method. The evolutionary distances were computed using the p-distance method, presented in the units of the number of amino acid differences per site.

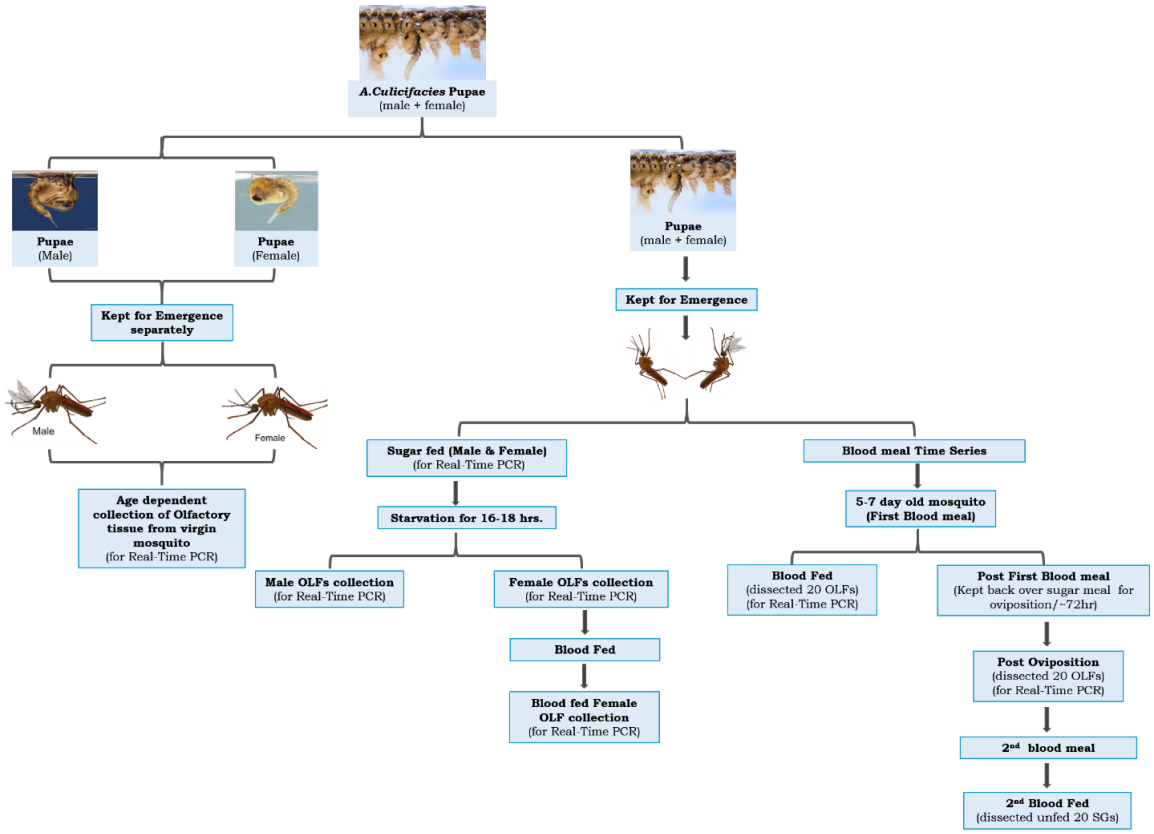

Figure 7.1: Technical designing and experimental work flow to demonstrate the possible role of *Ac-qtc* in mosquito behavioral regulation

7.2.3 Behavioral-cum-molecular assay

To track the possible role of *Ac-qtc* in mating behavior, an assay was designed (Figure 7.6) favoring sex-specific changes of the behavioral activities occurring in response to day/night cycle in the 5-6 days old mosquitoes. As per assay design, olfactory and reproductive tissues were collected from either virgin and/or mixed cage mosquitoes of both the sexes. For the assurance of mating success, first, an equal number of male and female virgin mosquitoes were mixed in a single cage at early morning (0800hr) and tissues collection were done at 1500 hr, 1900 hr, and overnight/0800 hr next morning from the same cage. To test and validate the completion of insemination process, the expression of two independent sperm-specific transcripts were profiled and compared in the spermathecae of adult female mosquitoes, where the sperms are stored after successful insemination. Previously characterized sperm-specific genes (AMS/FJ869235.1and MTS/FJ869236.1) of the mosquito *An. gambiae* [174] were queried to search and select sperm-specific homologs from the draft genome database of the mosquito *An. culicifacies*. The identified *Ac-ams* (ACUA010089) and *Ac-mts* (ACUA014389) transcripts sequences were used to design RT-PCR primers.

7.2.4 cDNA preparation and gene expression analysis

For cDNA preparation and relative gene expression profiling, exactly same protocol was used that mentioned in chapter 5. The sequence of the respective primers that were used for PCR amplification reaction is mentioned in the following table (Table 7.1).

Table 7.1: Primer sequence used in the study

Sl. No.	Gene Name	Primer sequence
1.	Ac-quick-to-court	Fw: 5' AGGTGCAGTTCATCGACA 3' Rev: 5'CTCCAGTCACCGATGTATCT 3'
2.	Ac_ams	Fw: 5'TCGCACGCATCAATAGAAAG 3' Rev: 5' TCTGCAATCTCTGCACTGCT3'
3.	Ac_mts	Fw: 5'GCTGCAAGGTGTGTCTTCCT 3' Rev: 5' AAAGCTCTCCACACGACCAC 3'

7.3 Results and discussion

7.3.1 Identification and molecular annotation of *Ac-qtc*

A unique transcript, encoding *Drosophila* homolog of Quick-To-Court (QTC) protein, was identified from blood fed olfactory transcriptome. BLASTX analysis of the partial 383 bp long transcript of *Ac-qtc* showed 59% identity with *Drosophila* homolog, but no putative conserved domain was identified. To retrieve full-length *An. culicifacies qtc* transcript, BLASTN analysis was performed against *An. culicifacies* genome as well as transcript databases, using the partial transcript as a query sequence. Comparative alignment analysis of the partial and predicted full-length transcript indicated that the identified 383 bp putative *Ac-qtc* transcript lacks both 5' and 3' sequences. The detail *in silico* analysis of 1536 bp long full-length *qtc* transcript (ACUA027268) encoding a 511 amino acid long protein showed coiled-coil domain signature at the 3' end of the sequence. *Quick-to-court* is a single copy gene, comprised of a 50 bp 5' UTR region followed by five exons and four introns followed by a 50 bp 3'UTR region as shown in Figure 7.2a. A comprehensive primary structural analysis of this full-length transcript (ACUA027268) revealed that it has four coiled-coils features and one conserved GRIP domain at the 3' end of the sequence (Figure 7.2b; Table 7.2).

Table 7.2: Domain features of *Ac-qtc* protein.

Sl No.	Feature Type	Start Site	End Site
1.	Coiled-coils (Ncoils)	118	146
2.	Coiled-coils (Ncoils)	275	310
3.	Coiled-coils (Ncoils)	332	367
4.	Coiled-coils (Ncoils)	396	417
5.	Prefolding Domain	285	363
6.	GRIP domain	463	504

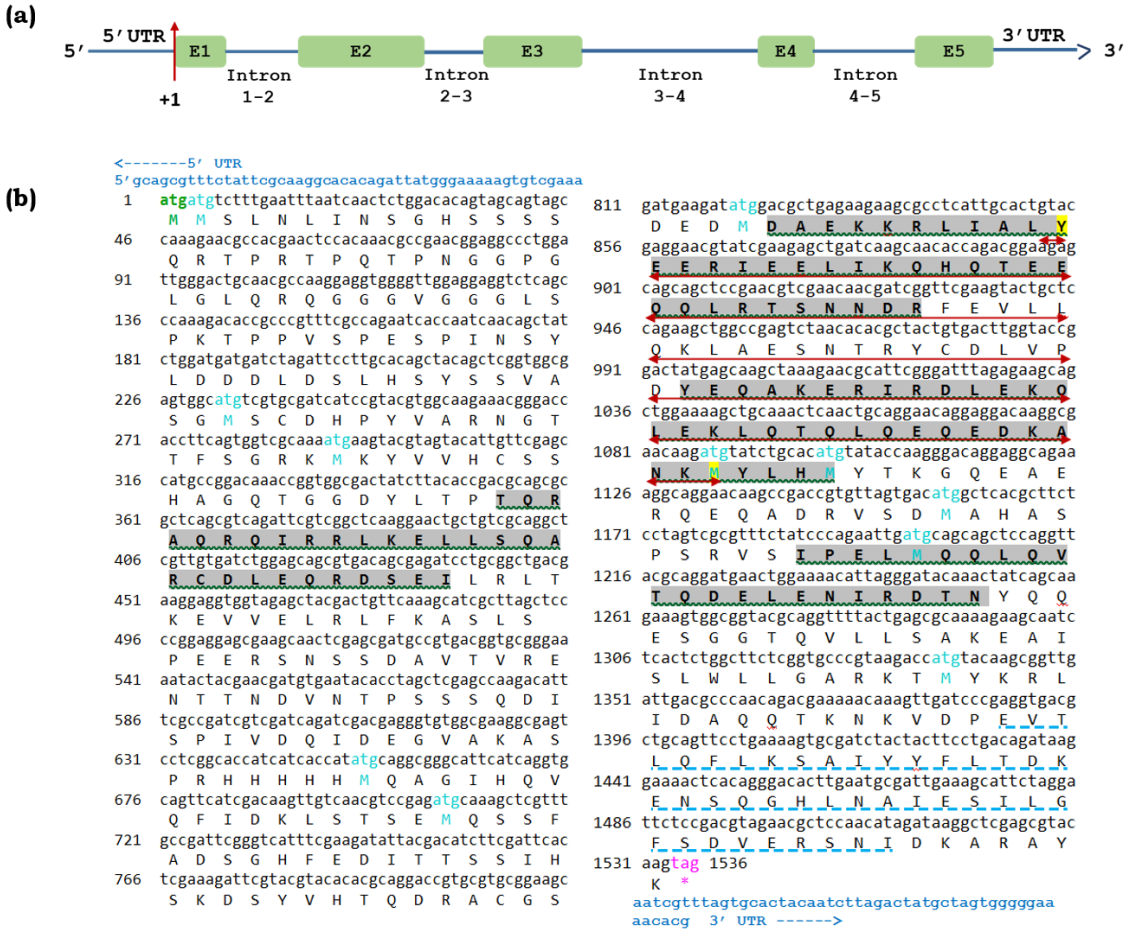

Figure 7.2: Genomic and molecular characterization of *An. culicifacies quick-to-court* gene. (a) Schematic representation of the genomic architecture of the mosquito *Ac-qtc*. Five green colored boxes indicate the introns and +1 mark the transcription initiation site. (b) Gene organization and molecular features of full-length *Ac-qtc*: The gene contains 1536 bp nucleotide, encoding 511 AA long peptides with four coiled-coils domains. Both 5' and 3'-UTR regions are highlighted (Yellow) and shown in bold & capital letters. The complete coding region of 511 amino acids starts from ATG/Methionine/green color, ending with TAG/Red/*. The different features are highlighted with different color code, viz. Coiled-coils domain (grey color and green underlined), Pre-folding domain (Red arrow) and GRIP-domain (sky blue dotted line).

7.3.2 Phylogenetic analysis of *Ac-qtc*

Multiple sequence alignment of *Ac-qtc* was performed with other mosquito species and *Drosophila* homologs using the CLASTALX2 software. This sequence alignment data showed a high degree of sequence conservation within the mosquito and other insect species (Figure 7.3a). The coiled-coils domains are highlighted with a red line. Phylogenetic analysis further supported that *Ac-qtc* is present in a cluster within the mosquito domain and have much greater similarity with other mosquito *qtc* than Drosophila homologs (Figure 7.3b).

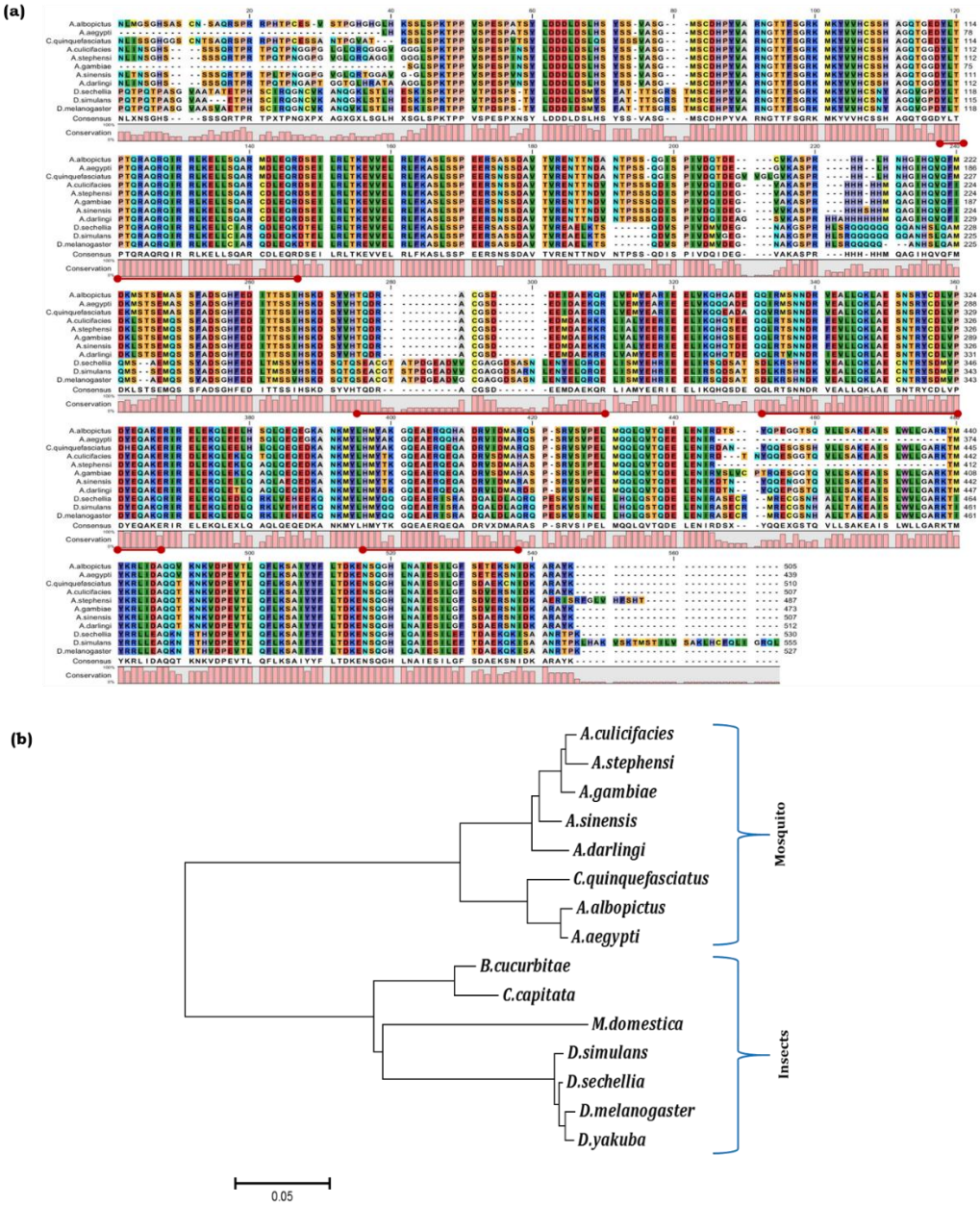

Figure 7.3: Sequence alignment and phylogenetic analysis of *Ac-qtc* gene. (a)Multiple sequence alignment of selected coiled-coil domain (marked with a green arrow) and (b) phylogenetic relationship of *Ac-qtc* with other mosquito species and *Drosophila*.

7.3.3 Mosquito tissue-specific expression analysis of *Ac-qtc*

An initial RT-PCR based expression analysis of *Ac-qtc* in the aquatic stages indicated that it constitutively expressed in all the stages of development, except in the egg of the mosquitoes (Figure 7.4a). Furthermore, sex and tissue-specific transcriptional profiling of *Ac-qtc* in the naive mosquitoes revealed that *qtc* gene is abundantly expressed in the olfactory tissue, brain and reproductive organs of both the sexes of mosquitoes (Figure 7.4b). The previous study in *D. melanogaster* also demonstrated that *Dm-qtc* is expressed in the olfactory organs, the central nervous system of both the sexes and male reproductive tract [169]. Interestingly, a relatively higher abundance of *qtc* gene in the olfactory tissue of male *An. culicifacies* indicated its possible involvement in the regulation of mosquito's mating behavior.

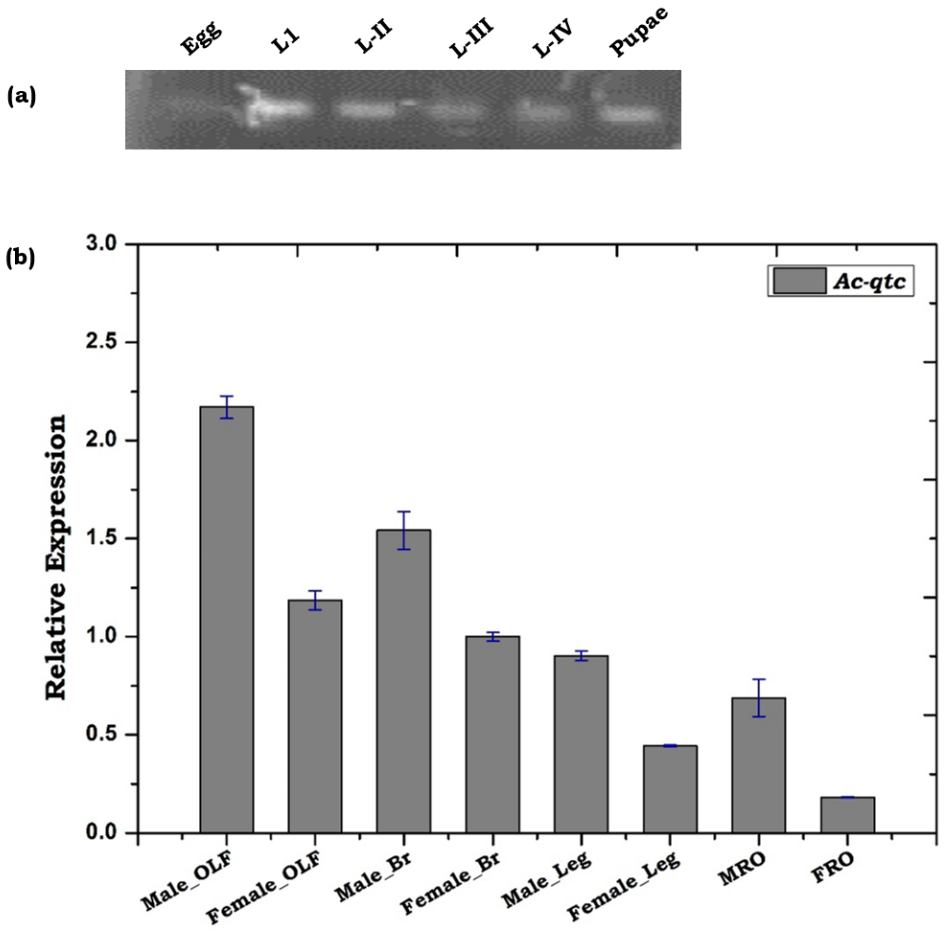

Figure 7.4: Transcriptional profiling of *Ac-qtc* transcript. (a) Developmental expression of *Ac-qtc* in *An. culicifacies* by RT-PCR. (b) Tissue and sex-specific relative expression analysis of *Ac-qtc* in the adult mosquito. Male OLF: Male olfactory tissue (Antennae, maxillary palp, and proboscis); Female OLF: female olfactory tissue; Male-Br: Male brain; Female-Br: female brain; Male-leg; Female-leg; MRO: Male reproductive organ; FRO: Female reproductive organ.

7.3.4 Age-dependent expression of *Ac-qtc*

An age-dependent transcriptional regulation of *Ac-qtc* was monitored in virgin male and female mosquitoes. Irrespective of the mosquito sexes, it showed an age-dependent enrichment of *Ac-qtc* at the highest level (~6- fold up-regulated/ $p \leq 0.004$) on the 5th day, when compared with 1-day old virgin mosquitoes (Figure 7.5b, c), followed by at least 2-fold down-regulation ($p \leq 0.0172$) on the 7th day. Interestingly after 7th-day *Ac-qtc* expression switched to up-regulation in male mosquitoes but remained constant after 5th day in case of female mosquitoes. A similar pattern of *Ac-qtc* gene expression was also observed in the possibly mated male mosquitoes (Figure 7.5d), which were collected from a mixed cage containing an equal number of male and female mosquitoes.

Although, it is not clear that how environmental guided non-genetic and/or genetic factors regulate the complex sexual behavioral events, our observations indicated that once male mosquitoes achieved the specific age of adulteration, the dysregulation of *Ac-qtc* by unknown mechanism may promote the courtship behavior. These results also corroborate with the previous findings in *Drosophila* where a mutation in *Dm-qtc* gene causes accelerated male-male courtship behavior [169]. A recent study by *Houot et. al.* also suggested that *qtc* and *shaker* genes, which are abundantly expressed in the neuro-olfactory system of *Drosophila*, decrease the ability to discriminate between the sex targets probably due to the declined perception to wild-type female pheromone [175].

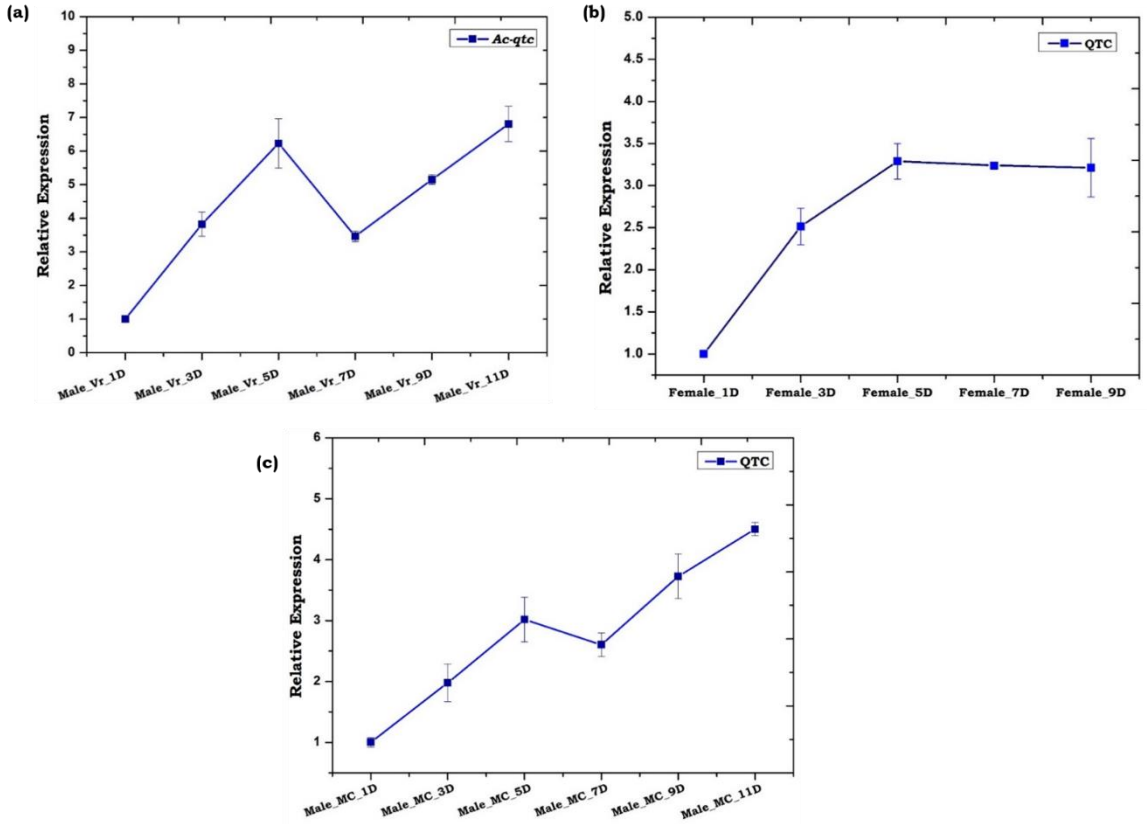

Figure 7.5: Sex-specific and age-dependent transcriptional response of *Ac-qtc* transcripts in the olfactory tissue of *An. culicifacies*. (a-b) Age-dependent transcriptional profiling of *Ac-qtc* in the virgin male and female mosquitoes. Male-Vr-1D: Male virgin mosquito of 1 Day old; Female-Vr-1D: Female virgin mosquito of 1 Day old. (c) Age-dependent relative expression analysis of *Ac-qtc* transcript in the mated mosquito. Male-MC-1D: Male mosquito of 1day old, collected from the mixed cage (MC) containing an equal number of male-female mosquitoes.

7.3.5 Natural dysregulation of *Ac-qtc* may promote mating success

An alternative interpretation, of the above argument, could be that a significant downregulation ($p \leq 0.0172$) of *Ac-qtc* in the 5-7 days old virgin adult male mosquitoes may be crucial for the auto-activation of courtship behavior in case of *An. culicifacies* mosquitoes. Except, to the limited knowledge that in most *Anopheline* mosquitoes, mating behavioral activities commenced by the onset of sunset, usually at 1700 hr which may continue till 2000 hrs. [171,176,177], the unavailability of any molecular marker for mating behavioral studies restricted in-depth understanding of the mating biology of mosquitoes.

Therefore, we hypothesize that the transcriptional modulation of *Ac-qtc* in response to dawn/dusk cycle must have a functional correlation with the mating success, especially insemination events where adult females receive and store the sperms in her spermathecae delivered by the male during copulation [178]. For experimental verification of this idea, two sperm-specific transcripts were first identified from the draft genome of *An.*

culicifacies, using previously characterized *An. gambiae ams* and *mts* gene as query sequences [174]. To validate sperm specificity, the primers designed against *Ac-ams* (ACUA010089) and *Ac-mts* (ACUA014389) were tested by RT-PCR in the male accessory glands (MAG) and spermathecae (SPT) collected from laboratory-reared 3-4 days old virgin male and female mosquitoes, respectively (Figure 7.6a). Next, to trace the sex-specific regulation of *Ac-qtc* in the olfactory tissue and predict possible functional correlation in mating success, we profiled and compared sperm-specific *Ac-ams/Ac-mts* genes expression in the male and female reproductive organs, collected two hours prior or later onset of the sunset as described in hypothesis figure described below (Figure 7.6b).

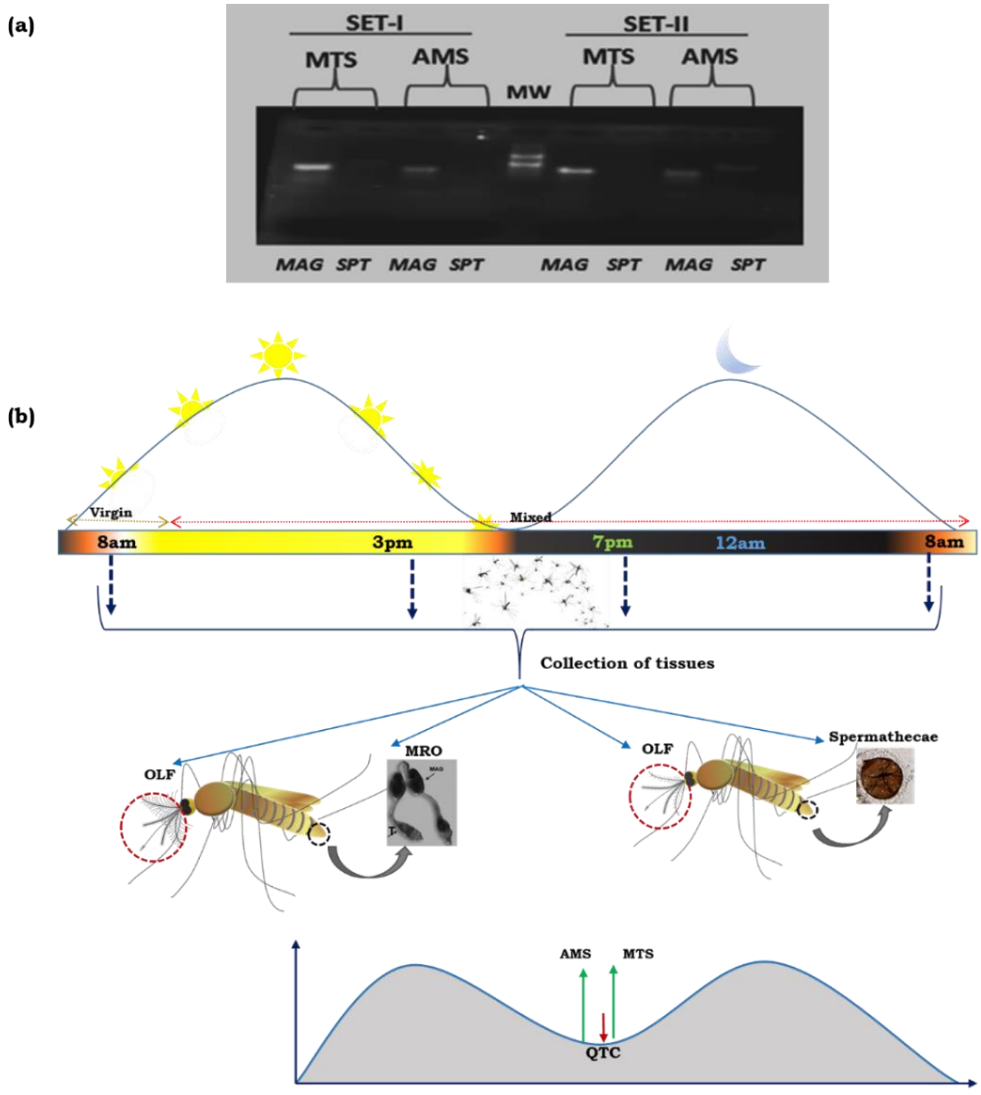

Figure 7.6: Primer validation and working hypothesis to decode *Ac-qtc* function in the mating biology of *An. culicifacies* mosquito. (a) RT-PCR based expression validation of *Ac-ams* and *Ac-mts* in MAG (Male Accessory Gland). (b) Pictorial presentation of the assay designed to correlate the function of *Ac-qtc* in the mating success of *An. culicifacies* mosquito.

When compared to the virgin counterpart, significant down-regulation of *Ac-qtc* was observed in the olfactory system of both the sexes at 1900 hrs. (Figure 7.7a). This data indicated that lower expression *Ac-qtc* might favor the increased mating frequency and active courtship engagement. Though exact molecular mechanism is yet to be explored, however, a significant modulation of *Ac-ams/Ac-mts* expression in the mated female spermathecae (Figure 7.7b) as well as male reproductive organs (Figure 7.7c) supported the idea that a natural dysregulation of *Ac-qtc* in the late evening i.e. 1900 hr. may favor the copulation process by facilitating the release of unknown sex driving factors for successful insemination event completion in the copulating couples.

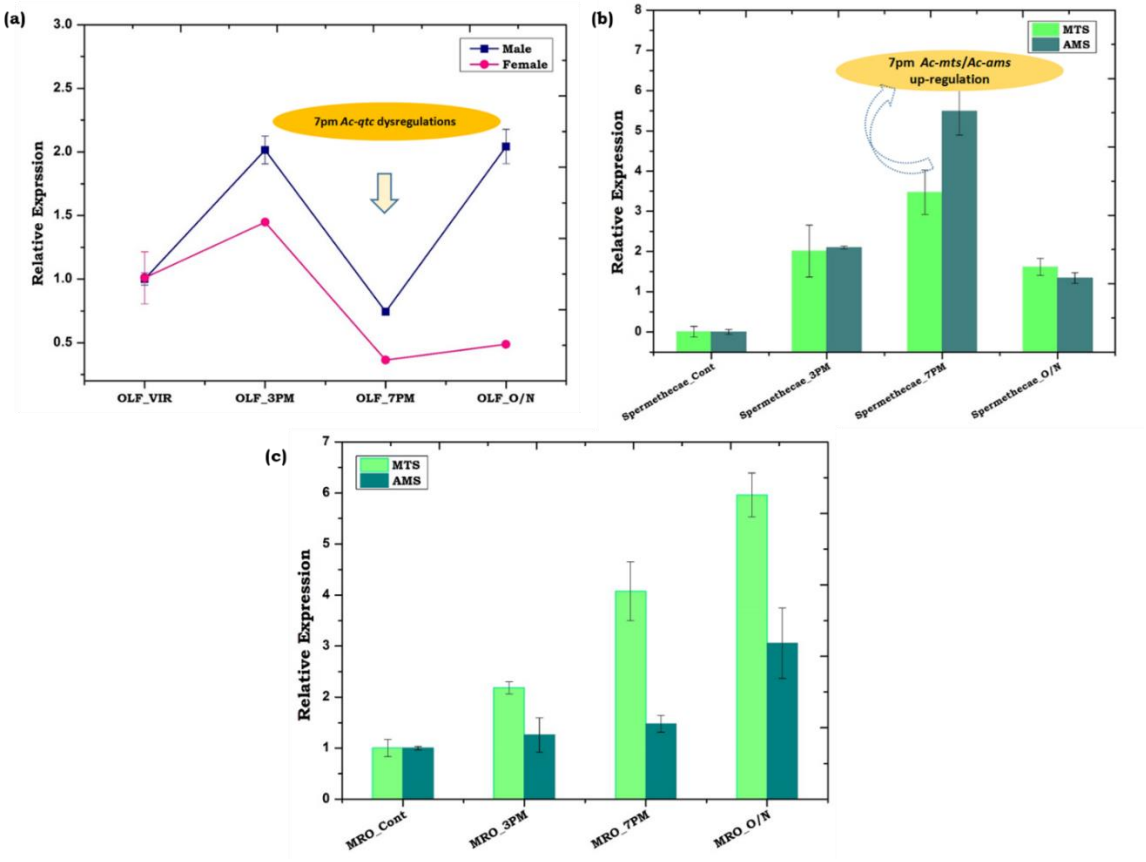

Figure 7.7: Natural dysregulation of Ac-qtc favor successful insemination. (a) Sex-specific transcriptional profiling of *Ac-qtc* at different circadian time in the olfactory tissues of the mosquito *An. culicifacies*; (b) Transcriptional response of *Ac-mts* and *Ac-ams* in the spermathecae of *An. culicifacies* at different circadian time. (c) Transcriptional Response of *Ac-mts* and *Ac-ams* in male reproductive organ (MRO) at different circadian time.

7.3.6 Nutritional status dependent expression of *Ac-qtc* in the mosquitoes

Feeding and mating are two mutually exclusive but interdependent behavioral properties of any living animal that facilitate their survival and reproductive success synergistically. The molecular basis of the sex-specific regulation of these conflicting behavioral demands

remains largely unknown. Current studies in *Drosophila* suggested that food odor and sex-specific pheromone signals in the neuro-olfactory system work interactively to drive both the meal and/or mate attraction [175]. Thus, to test whether *Ac-qtc* has any sex-specific relation to the nutritional status of naïve mosquitoes, the relative expression of *Ac-qtc* transcript in starved and sugar fed mosquito were examined and compared in both the sexes. To perform this experiment, olfactory tissues were collected from 5-6-day old sugar fed and 16-18 hrs starved mosquitoes. Relative gene expression analysis indicated that starvation did not affect the abundance of *qtc* transcript, but showed a two-fold up-regulation ($p \leq 0.03$) in response to immediate i.e. 30 min – 1 hr. post blood feeding (Figure 7.8). Together these data suggested that *Ac-qtc* may not be essential for regulating mosquito sugar feeding associated behavior but may have an important role in the regulation of host-seeking/blood-feeding behavior in the adult female mosquitoes.

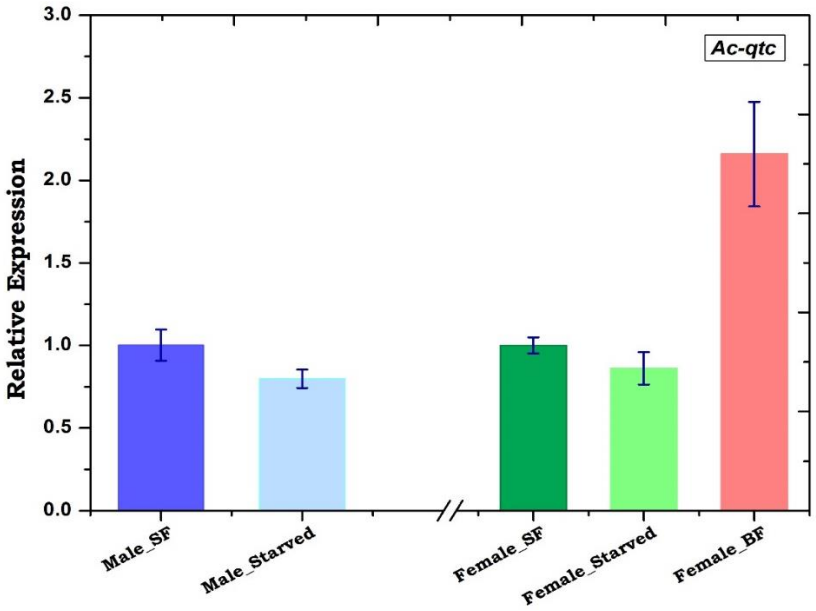

Figure 7.8: Effect of starvation on *Ac-qtc* expression. Both male and female mosquitoes were kept starved for 16-18 hr and the olfactory tissue was collected for *qtc* expression study. Additionally, blood meal was provided to female mosquitoes after starvation.

7.3.7 Effect of blood meal in the expression of *Ac-qtc* in adult female mosquito

To further evaluate *Ac-qtc* role in response to blood feeding behavior of female mosquitoes, a blood meal time series experiment was performed as described earlier (Chapter 5). *Ac-qtc* expression showed a gradual increase in abundance till 5th day, but significant (2.5 fold / $p \leq 0.03$) downregulation was observed on the 7th day in the naive unfed adult female mosquitoes. This expression pattern is similar to the adult male mosquitoes (Figure 7.9), which may facilitate and make the courtship behavior successful in both the sexes.

Although it is not clear whether the first blood meal transiently and/or completely pauses re-mating events, interestingly, a consistent up-regulation of *Ac-qtc* just after blood feeding (within 30 min) till 30 hrs post blood meal (Figure 7.9), indirectly suggested that the adult female mosquito may not seek any courtship event at least for the first 30 hrs post blood meal. Furthermore, a continuous sharp downregulation (~4 fold) of *Ac-qtc* till 72 hrs post first blood meal remains questionable, whether *Ac-qtc* promotes host-seeking behavior for second blood meal and/or initiate mate partner finding. Significant up-regulation (~1.3 fold; Fig. 7.9) of *Ac-qtc* after oviposition and prior to second blood meal also supported the hypothesis. Second blood meal again rapidly downregulated the *Ac-qtc* expression, when examined 30hr post blood fed in the olfactory tissue. Together these data indicate that *Ac-qtc* may have a unique role in driving dual mode of behavioral responses possibly to meet the conflicting demand of sexual mate partner and/or suitable vertebrate host finding for blood feeding.

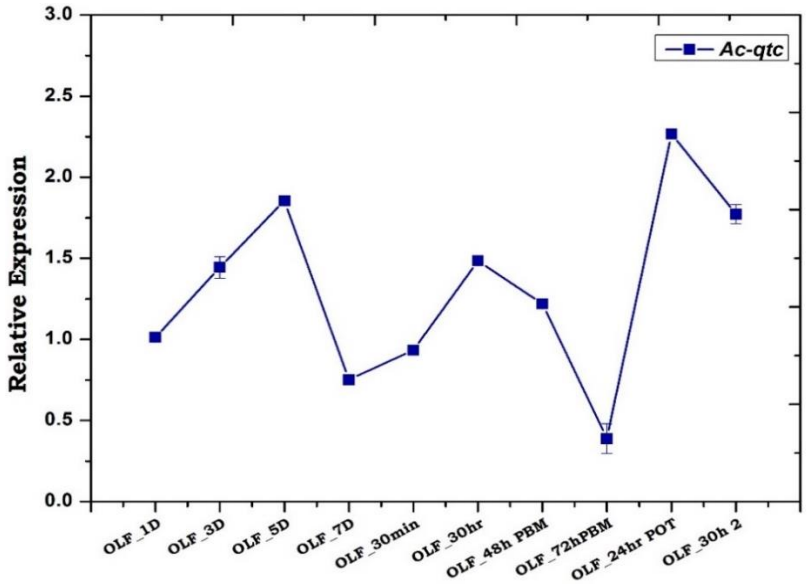

Figure 7.9: Transcriptional behavior of *Ac-qtc* in two consecutive blood meal follows up. OLF-1D – OLF-7D: Olfactory tissue from 1Day -7 Day old female; OLF-30min: Olfactory tissue collected from 30min post blood-fed mosquito; OLF-30hr: Olfactory tissue collected from 30hr post blood-fed mosquito; OLF-48hr: Olfactory tissue collected from 48hr of post blood-fed mosquito; OLF-72hr: Olfactory tissue collected from 72hr post blood-fed mosquito; OLF-24hr POT: Olfactory tissue collected from 24hr of post oviposition of mosquito; OLF-30hr 2: Olfactory tissue collected from 30hr of 2nd blood meal.

7.4 Conclusion

Examination of the sex-specific transcriptional regulation of an olfactory derived unique transcript *Ac-qtc* was performed through the comprehensive molecular approach and its possible role was predicted with 'food choice' and/or 'mate choice' behavioral performance (Fig. 7.10). This is the first molecular evidence stating that Ac-QTC proteins may have a dual mode of action in the regulation of a cluster of mosquito olfactory genes that are linked to mating success and/or blood feeding in adult female mosquitoes.

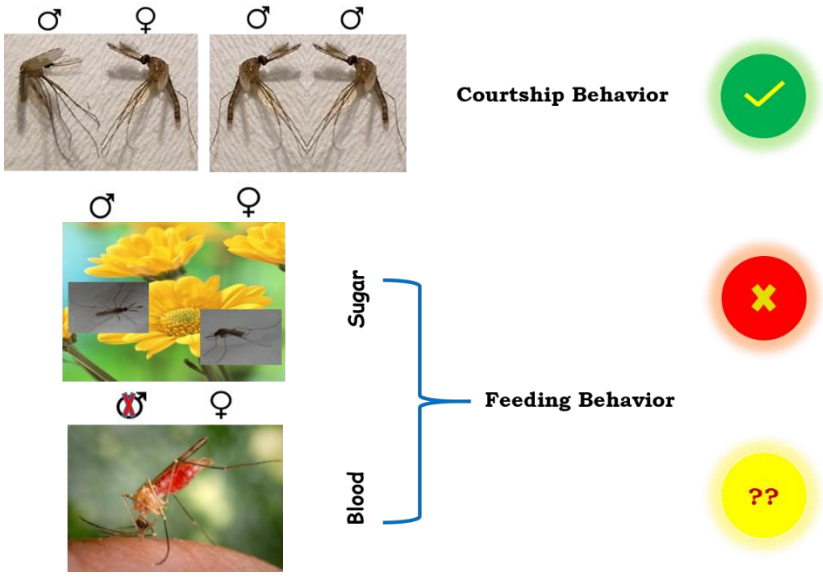

Figure 7.10: A proposed hypothesis for the possible function of *quick-to-court* gene in the mosquito. Photo credit to James Gathany for the blood-fed mosquito picture.

Chapter 8: Exploring the Possible Role of *Ac-attractin* Gene (*Ac-atrn*) in Neuro-olfactory Regulation of *Anopheles culicifacies* Mosquito

8.1 Introduction

Attractin is a CUB family of type I glycosylated protein having multiple domains. It is widely expressed in different types of tissues in the animal body and facilitate cell-cell adhesion [179,180]. It also acts as a guidance molecule in regulating multiple patho-physiological processes [179]. *Attractin* (*Atrn*) primarily exists in two forms, a transmembrane and a secreted form [181] and both of them contains two epidermal growth factor domains (EGF), a CUB domain, a C-type lectin domain, and two laminin-like epidermal growth factor domains. Membrane type *Atrn* has a novel function in myelination and thus prevent neuronal damage during oxidative stress [180]. Though humans possess both the membrane and the secreted form of *attractin,* but only the membrane type have been identified in mice, where it also found to regulate the proper functioning of sperm in the testes [182]. In vertebrates, an age-dependent progressive loss of function of *attractin* gene causes testis vacuolation and a decline in sperm function [182]. In invertebrates, it was first identified as water-borne pheromone from the mollusk *Aplysia californica,* which is synthesized and secreted by the female reproductive organ to guide and attract sperms, and facilitate sperm-egg interaction for successful fertilization, within a distance of at least ten meters [183].

Discovery of an *attractin* homolog from the olfactory tissue of *Anopheles culicifacies* mosquito prompted us to investigate its possible role in diverse behavioral responses e.g. feeding, mating and other non-genetic stresses. A comprehensive *in-silico* analysis and extensive transcriptional profiling in response to feeding, mating, and other stress responses provide an evidence that *Ac-attractin* may have an important role in the management of neuro-olfactory regulation and stress management, enabling mosquito's successful survival.

8.2 Material and methods

The same protocol was followed for mosquito rearing, tissue collection, RNA extraction and molecular gene expression analysis as described in chapter 5. Only the unique experiments that are designed to characterize Ac-*atrn* gene are described below.

8.2.1 External stress response of first instar larvae

To understand the response of *Ac-atrn* under the external stressed condition, the first instar larvae (~80-100) were kept overnight at 4^0C. For heat treatment, the (~80-100) larvae were exposed to 42^0C for 4 hours. Then to track the *Ac-atrn* expression under the nutritional stressed condition the fresh hatched first instar larvae were taken and divided into three groups each comprising ~50 larvae. One group of larvae were collected immediately after hatching as control batch, the second group of larvae was kept overnight in starvation, without any food supply. And the third group was provided with 20-30 mg of a mixture of fish food and dog biscuit. Next day, thirty starved larvae were collected in trizol and then the food was provided to remaining starved larvae to recover from the nutritional stress. After 24 hrs. of maintenance of the respective larvae with food, they were collected in trizol for relative gene expression analysis.

8.2.2 External stress response of adult mosquitoes

To expose the adult male and female mosquitoes with extrinsic stress, the adult mosquitoes were kept starved for overnight. Next day, the olfactory and brain tissue were dissected from ~25-30 starved and their respective sugar-fed mosquitoes. Further, a detailed time course of the starvation assay was performed and the brain tissue was dissected and collected in trizol to analyze the *Ac-atrn* expression in brain tissue during starvation.

8.2.3 Behavioral and molecular assay

To track the possible role of *Ac-atrn* in mating behavior, an assay was designed favoring sex-specific changes of the behavioral activities occurring in response to day/night cycle in the 5-6 days old mosquitoes. As per assay design, we collected olfactory and reproductive tissues from either virgin and/or mixed cage mosquitoes of both the sexes. For the assurance of mating success, we mixed an equal number of male and female virgin mosquitoes in a single cage at early morning (1000 hr) and collected tissues at 1700 hr and overnight/1000 hr next morning from the same cage.

8.2.4 cDNA preparation and gene expression analysis

For cDNA preparation and relative gene expression profiling, exactly same protocol was used that mentioned in chapter 5. The sequence of the *Ac-atrn* primers that were used for

PCR amplification reaction is mentioned below:

Ac-atrn_Fw: 5'TATCGAGAAGCCACTGTTTT 3'

Ac-atrn_Rev: 5'TGACGAGGAGTAAAAGGAAA 3'

8.3 Results & discussion

8.3.1 Identification, annotation and molecular characterization of *Ac-atrn*

During annotation of olfactory tissue transcriptome data, a 1070bp long unique transcript was discovered encoding putative *attractin* protein (Accession# MF599469). Initial BLASTX analysis against the NCBI NR database showed significant hits to the predicted putative *attractin* like proteins of multiple mosquitoes, insects and other invertebrate species. To retrieve full-length *Ac-atrn* transcript, BLASTN analysis was performed against the genome predicted transcript database of the mosquito *An. culicifacies,* which is available at www.vectorbase.org. This analysis predicted a 3942 bp long full-length transcript (ACUA004165-RA), encoding 1313 amino acid long peptide. A detailed *in silico* analysis of this full-length transcript, revealed that *Ac-atrn* is a single copy gene having six exons and five introns with 50bp flanking sequences at the 5' upstream region and 50bp 3' overhang at the downstream region (Figure 8.1a). A functional prediction analysis unraveled that putative *Ac-atrn* is a multi-domain protein containing at least five domains. These include two calcium binding EGF domain, one cysteine-rich Plexin repeat-PSI domain, one nitrile specific-PLN01293 and one extracellular CUB domain (Figure 8.1b).

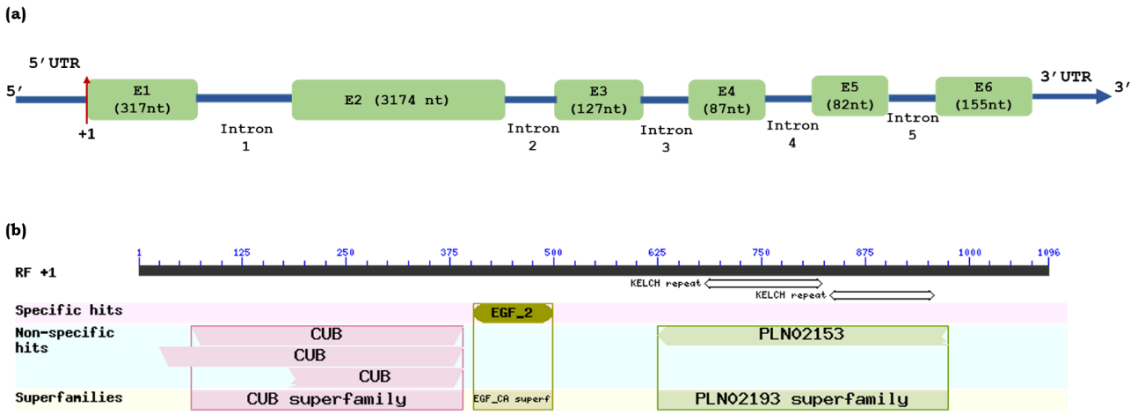

Figure 8.1: Molecular analysis of *An. culicifacies attractin (Ac-atrn)* gene. (a) Schematic representation of the genomic architecture of the *Ac-atrn gene.* Five green colored boxes indicate the exons, blue line denotes the introns and +1 mark the transcription initiation site. The size of the exons and introns correspond to the size of the boxes and lines. (b) Domain architecture of *Ac-atrn* gene.

8.3.2 Multiple sequence alignment and phylogenetic analysis of *Ac-atrn*

Multiple sequence alignment analysis of the *Ac-atrn* gene using CLASTALX2 software, showed a high degree of sequence similarity in the predicted domains named EGF, a cysteine-rich repeat and CUB domain (Figure 8.2a). This sequence conservation suggests a similar kind of functions of *attractin* gene in the mosquitoes. However, the investigations demonstrating the functional role of *attractin* in immunity, neuro-physiology, and reproduction are exclusively limited to vertebrates like human and mice [179,180,182,184]. *Aplysia californica* is the only invertebrate species where *attractin* guides sperm motility towards distantly located eggs for successful fertilization [183,185]. But, its role has not been investigated in any of the mosquito species so far.

Therefore, to predict the possible evolutionary relationship of the mosquito *attractin* gene, a comprehensive phylogenetic analysis was conducted using the MEGA6 software. Phylogenetic data showed a conserved relationship among blood feeding as well as non-blood feeding insects, and animals, whereas invertebrate *Aplysia californica* and human *attractin* sequences appeared as an outgroup clustering (Figure 8.2b). This analysis hypothesized that *attractin* gene may have a similar multi-physiological role of in mosquitoes. To test this hypothesis, we extensively profiled the transcriptional response of *Ac-atrn* gene under the distinct physiological status of the mosquito *An. culicifacies*.

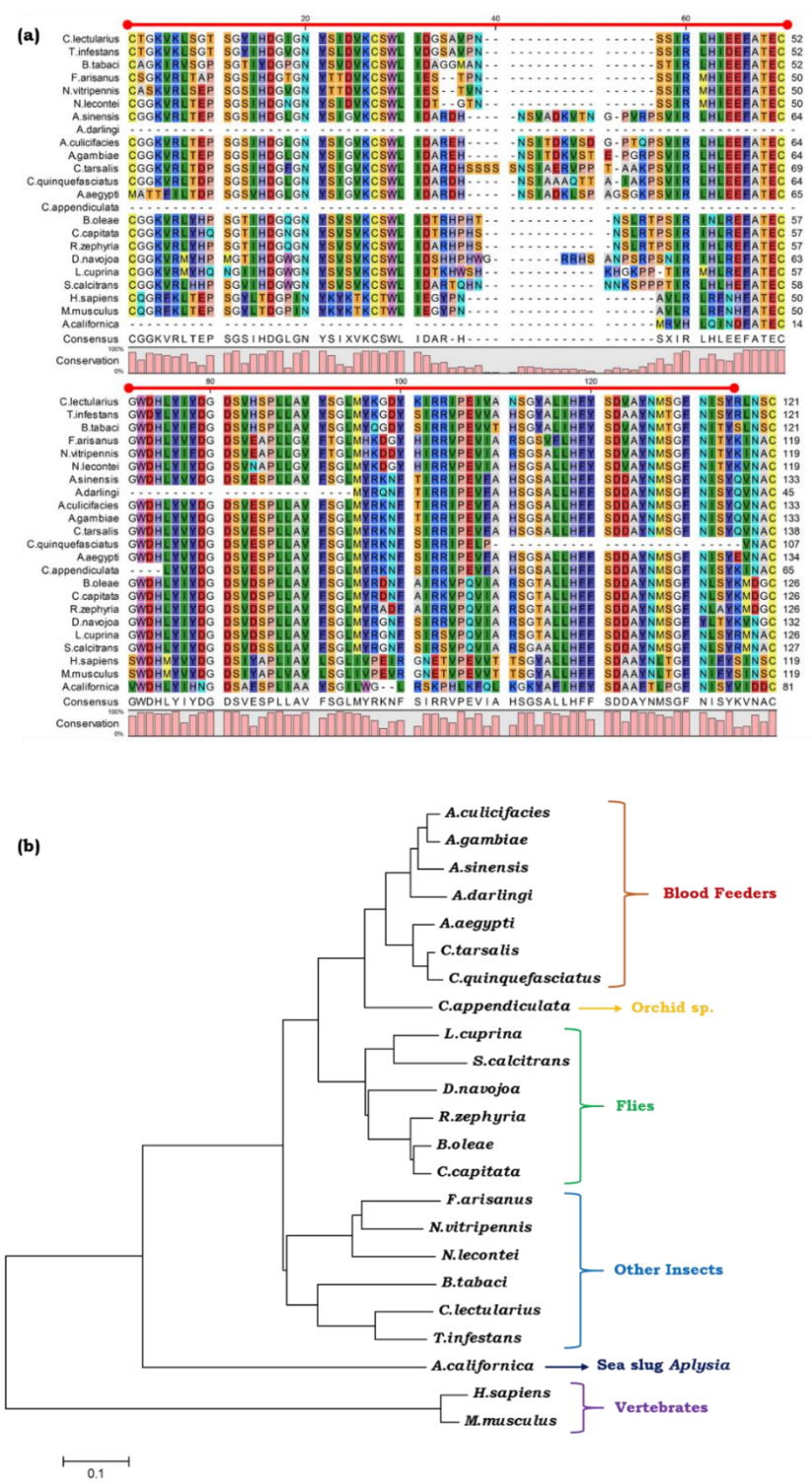

Figure 8.2: Sequence alignment and phylogenetic analysis of Ac-atrn gene. (a) Multiple sequence alignment of a segment of *An. culicifacies attractin* gene with other mosquitoes, flies, invertebrates and vertebrates' homologs showed high degree of conservation in the amino acid sequence. One of the CUB domain are highlighted with red line. (b) Phylogenetic relationship of *Ac-attractin* indicates that *An. culicifacies attractin* is clustered within the mosquito domain and have much greater similarity with mosquito *attractin* than flies and other insects.

8.3.3 Food supplement induces *attractin* response in mosquito's larvae

To unravel whether *Ac-atrn* has any role during the aquatic development of the mosquito, a developmental stage-specific relative gene expression analysis was performed. A real-time PCR analysis showed relatively higher expression of *Ac-atrn* in the young L1 larvae when compared to egg and other developmental stages (Figure 8.3a). This data indicated that an increase in *attractin* expression in emerging young larva may be important to taste, smell and move towards food sources. Ten hours of food deprivation did not alter *attractin* expression in the larvae, suggested that nutritional stress did not have any effect on the expression of *attractin* in the larvae. However, surprisingly, a two-fold ($p< 0.01$) up-regulation of *Ac-atrn* level was observed in the naïve as well as the starved larvae, when they were provided with fresh food prior and after starvation, respectively (Figure 8.3b). Together, these data suggested that a food supplement may stimulate *Ac-atrn* expression, possibly to regulate the larval movement towards the food source. This may be one of the unique features of *attractin* protein, having the property of water-mediated chemical communication (pheromone-mediated) ability, similar to sperm-egg interaction in *Aplysia calfornica* [183].

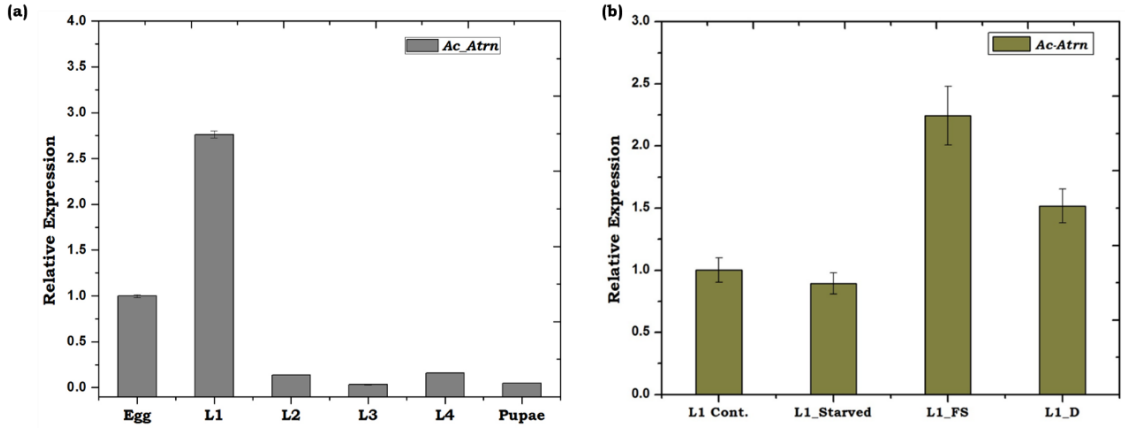

Figure 8.3: Transcriptional profiling of *attractin* gene in *An. culicifacies* developmental stages. (a) Real-Time PCR mediated developmental expression of *Ac-atrn* in *An. culicifacies*. (b) Relative expression analysis of *Ac-atrn* under food stressed conditions in the first instar larvae.

8.3.4 Cold stress seizes *attractin* expression

Next, to test whether external/environmental stress influences the *Ac-atrn* expression, the young larvae were exposed to overnight cold stress and compared its expression with unstressed larvae. Though cold stress did not affect the survival of the larvae, but the depletion of *Ac-atrn* to a negligible level ($p<0.002$) was observed in the cold treated larvae (Figure 8.4). We also observed that cold treatment temporarily arrested the motility of the larvae, which was recovered to the normal active stage when kept back at room temperature for 3-4 hrs. Along with the recovery of larval movement, *Ac-atrn* expression also reached

to normal level after 3-4 hrs of the recovery phase. However, 4-5 hrs. of heat exposure of the larvae at 42^{o}C did not alter *Ac-atrn* expression significantly ($p<0.1$). Though it is yet to be clarified the exact role of *Ac-atrn* in the regulation of thermal stress, however, these data indicated that cold stress may temporarily arrest *attractin* expression, a response necessary to cease the motility in order to minimize the energy loss (Figure 8.4).

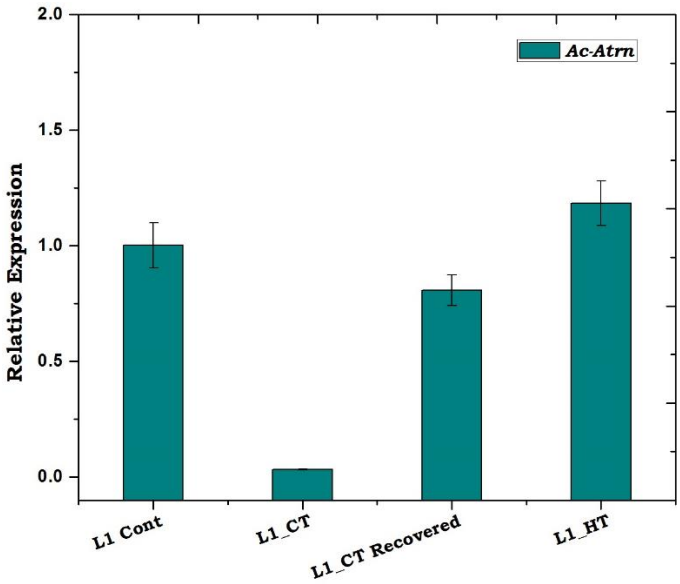

Figure 8.4: Differential gene expression analysis of *Ac-atrn* gene in the first instar larvae under temperature stressed conditions. L1 Cont: L1 larvae control set without any treatment; L1_CT: Cold Treated L1 Larvae; L1_CT_Recovered: Recovered Cold Treated L1 larvae; L1_HT: Heat Treated L1 Larvae.

8.3.5 Nutritional stress and *attractin* response in the adult mosquito's olfactory system

To evaluate the tissue specificity of *Ac-atrn* gene in naïve adult mosquitoes, different tissues viz. olfactory tissues, brain and reproductive organs of both male and female mosquitoes were collected in trizol at the same time and cDNA was prepared by following the standard protocol. Further, relative gene expression analysis was performed by real-time PCR. This study indicated that *Ac-atrn* gene constitutively expressed in the olfactory tissues, central nervous system and the reproductive organs of both male and female mosquitoes (Figure 8.5a). But, ~2.5fold higher level of expression was observed in the neuro-olfactory system than the reproductive tissues of both the sexes of naïve mosquitoes (Figure 8.5a), suggested its possible role in mosquito's behavioral biology and stress management. To unravel the function of *Ac-atrn* in olfactory and neuronal regulations, first, we compared a relative gene expression between sugar and blood-fed mosquitoes olfactory system, however, the data did not show any significant alteration in the *Ac-atrn* expression (Figure 8.5b). Furthermore, a 24hrs of starvation also had no effect on the expression level of *Ac-atrn* gene (Figure 8.5c) in the olfactory tissue of adult mosquitoes

of both sexes. Together these data indicated that *attractin* protein may not be associated with the regulation of feeding behavior in adult female mosquitoes.

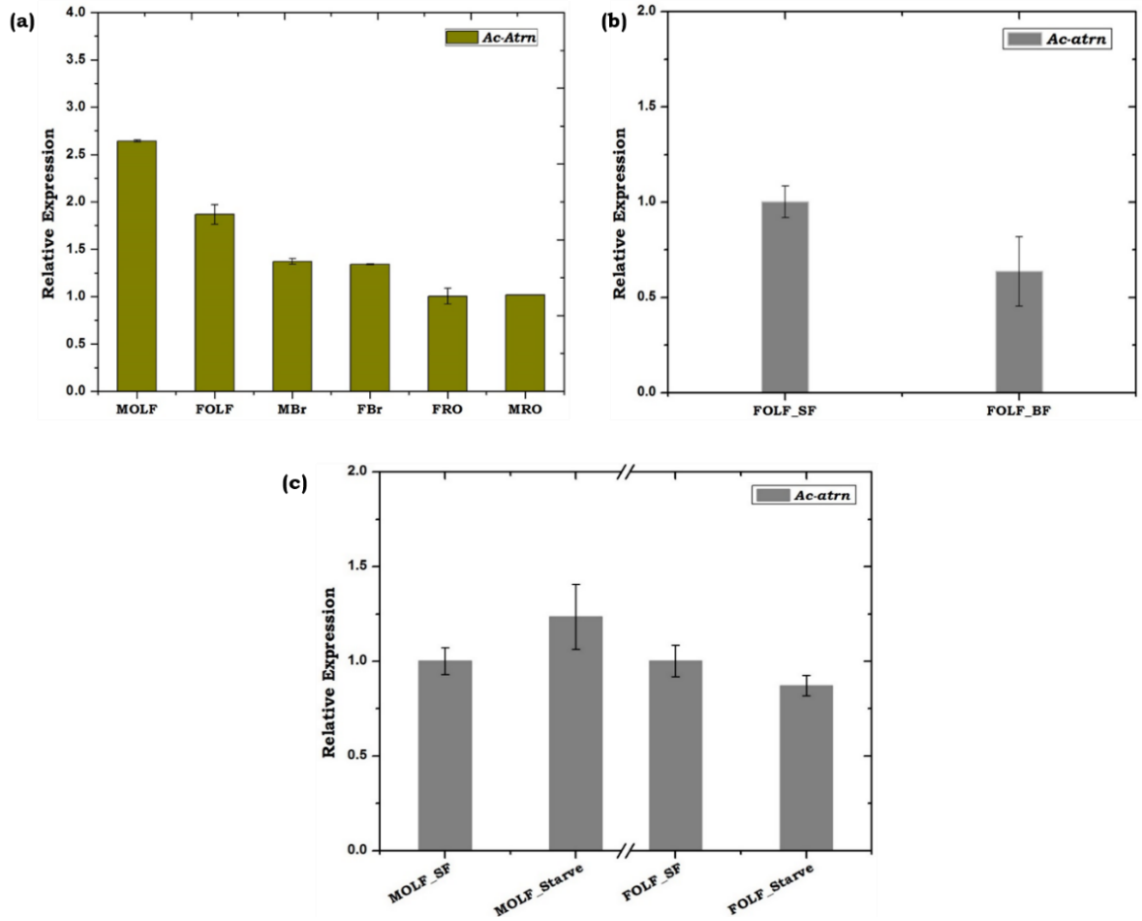

Figure 8.5: Sex and Tissue-specific transcriptional behavior of *Ac-atrn*. (a) Tissue-specific relative expression analysis of *Ac-atrn*. MOLF: Male olfactory tissue (Antennae, maxillary palp and proboscis); FOLF: female olfactory tissue; MBr: Male brain; FBr: female brain; MRO: Male reproductive organ; FRO: Female reproductive organ. (b) *Ac-atrn* expression pattern in sugar fed and blood fed olfactory tissues. FOLF_SF: sugar fed female olfactory tissue; FOLF_BF: Blood fed female olfactory tissue. (c) Transcriptional response of *Ac-atrn* in the olfactory tissues of both male and female mosquitoes under food deprived condition. MOLF_SF: sugar fed male olfactory tissue; MOLF_starve: 24hrs. starved male olfactory tissues (Same for the females).

8.3.6 Starvation modulate *attractin* expression in the adult brain

Although, nutritional stress did not alter *Ac-atrn* expression in the olfactory system, surprisingly, a time-dependent starvation significantly modulated *Ac-atrn* expression in the brain tissue of adult mosquitoes of both sexes (Figure 8.6a, b). The male mosquitoes brain showed an early transcriptional response (6 hrs. after starvation) of *Ac-atrn* gene ($P<0.0001$) under nutritional stressed condition (Figure 8.6a), whereas, female mosquitoes brain showed a delayed elevation of *Ac-atrn* at 30hrs of starvation ($p<0.0001$) (Figure 8.6b). These data indicated that male brains are more susceptible to starvation-induced neuronal damage as compared to female brains because in theses experiment, we also observed that the mortality rate of male mosquitoes is much higher than their female counterpart (Figure 8.6c). A substantial body of the literature suggested that human and other vertebrate brain is the highly metabolic organ in the body which consumes a large amount of energy in the form of nutrition/food [186]. Although, the brain is highly susceptible to oxidative damage due to the abundance of oxidizable material in the plasma membranes of neural cells. However, food deprivation has an added value which causes a failure in the oxidative stress management and thus leads to brain cells degeneration and death [187].

Thus, taken together, we hypothesize that an early up-regulation of *Ac-atrn* in the male brain may be an attempt to protect the brain cells from fasting-induced oxidative damage and consequently neuronal degeneration and death [187]. Whereas, >10-fold elevation of *Ac-atrn* in the female brain during a later stage of starvation suggested that female mosquitoes can survive a longer period of time (Figure 8.6c) without any food source and thus are more adaptive to adverse environmental conditions which favor its evolution and existence.

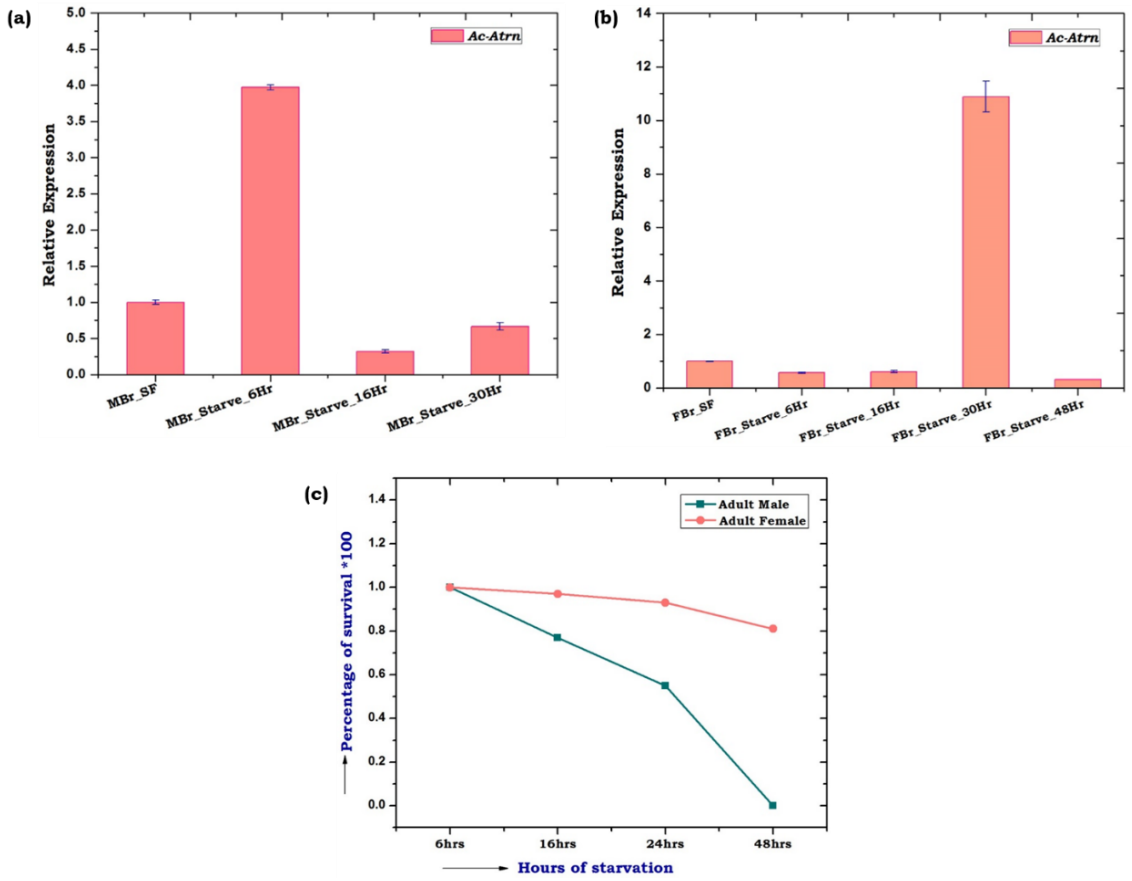

Figure 8.6: Sex and Tissue-specific transcriptional behavior of *Ac-atrn* during nutritional stress. (a) A time-dependent transcriptional profiling of *Ac-atrn* in the brain tissues of male *An. culicifacies* mosquitoes under food deprived condition. MBr_SF: Male brain dissected from sugar-fed mosquitoes; MBr_Starve_6Hr: Male brain dissected after 6hrs of starvation (Same in case of other time points). (b) A time-dependent transcriptional profiling of *Ac-atrn* in the brain tissues of female mosquitoes under food deprived condition. FBr_SF: female brain dissected from sugar-fed mosquitoes; FBr_Starve_6Hr: Female brain dissected after 6hrs of starvation (Same in case of other time points). (c) Survival curve of 3-4 days old adult male and female mosquitoes under food-deprived conditions.

8.3.7 Age and sex-dependent olfactory response of *attractin*

The previous literature review suggested that *attractin* is a protein with multi-functional properties. Thus, to test the multifaceted role of *Ac-atrn* in regulating mosquito's behavioral biology, at first we tested whether age-dependent maturation affects *Ac-atrn* responses. To examine this, an age and sex-specific relative expression analysis of *Ac-atrn* were performed in the olfactory and brain tissue of *An. culicifacies* mosquito. A significant and continuous increase (~6 fold for female OLF and ~3.5 fold for male OLF) in *Ac-atrn* expression till the 7th day was observed in the olfactory tissue of both virgin male and female mosquitoes (Figure 8.7a, b). Together these data suggested that olfactory *Ac-atrn* may have an important role in the regulation of mosquito behavioral events. However, as

compared to the olfactory tissue brain did not show any significant modulation *Ac-atrn* level, except an initial change in expression in the aging adult female mosquitoes (Figure 8.7c, d), suspecting its possible role in attaining the adulteration age. An age-dependent increase of *Ac-atrn* in the olfactory tissue of both the sexes, therefore, further prompted us to test the possible role of *Ac-atrn* in mating behavior.

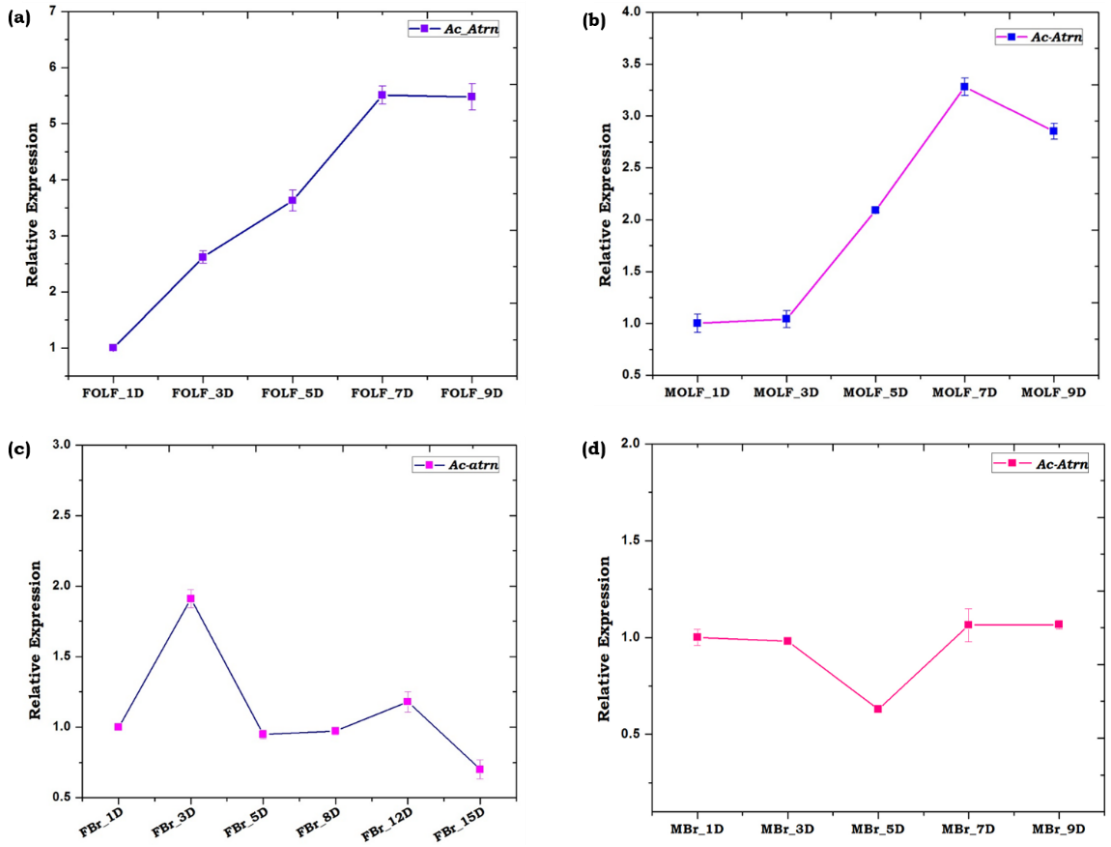

Figure 8.7: Age-dependent transcriptional response of *Ac-atrn* transcript in male and female *An. culicifacies* mosquitoes. (a) Age-dependent relative transcriptional regulation of *Ac-atrn* in female mosquito olfactory system. (b) Transcriptional response of *Ac-atrn* in male mosquito according to their age. (c) Age-dependent relative transcriptional profiling of *Ac-atrn* in female mosquitoes' brain. (d) Age-dependent relative transcriptional profiling of *Ac-atrn* in male mosquitoes' brain.

8.3.8 Role of *Ac-atrn* in mosquito's mating behavior

A circadian dependent transcriptional profiling indicated that mating status did not alter the *Ac-atrn* expression in the reproductive tissue of both the sexes (Figure 8.8a). However, a significant (>2.5 fold) change in the *Ac-atrn* level of mated mosquito's olfactory system provides an evidence that *attractin* may facilitate pheromone guided male-female courtship behavior (Figure 8.8b). For a successful mating, mosquitoes need to deal the complex

events of swarm formation and courtship engagement, ending with successful insemination; a process possibly guided by natural dysregulation of quick-to-court protein in the olfactory system of the mosquitoes (Chapter 7). Though it is yet to be clarified that how active swarm formation and courtship engagement is guided, but our current data suggested that *Ac-atrn* may have a key role to attract the couples during swarm formation, which is actively commenced on the onset of the sunset (17:00 hrs.).

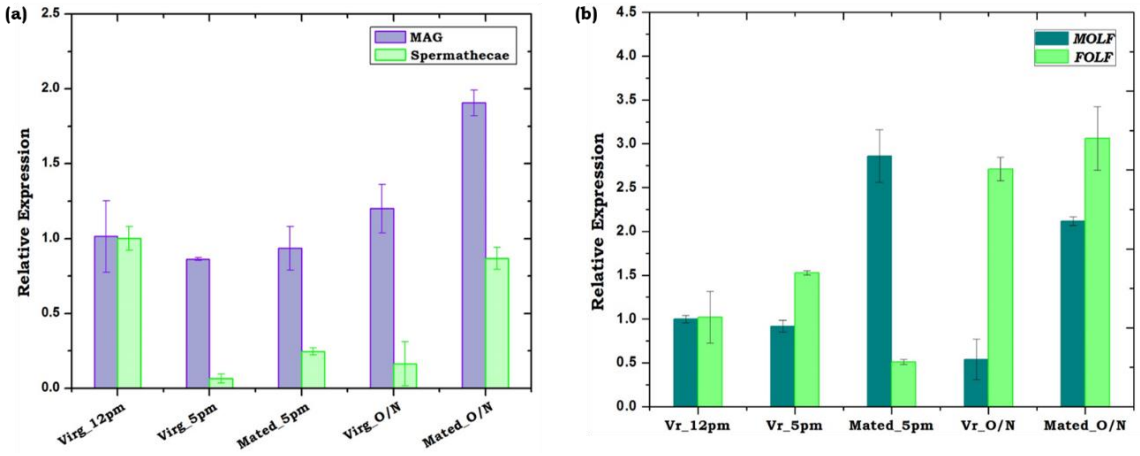

Figure 8.8: Transcriptional response of *Ac-atrn* gene according to mating status. (a) Circadian time-dependent and the mating status dependent expression pattern of *Ac-atrn* in the reproductive organ of both male and female mosquitoes. Virg_12pm: Virgin mosquitoes dissected at 12 pm; Mated_5pm: Mated mosquito dissected at 1700 hr.; MAG: Male accessory gland; Virg_O/N and Mated_O/N: Virgin and mated mosquito dissected after overnight exposure to each other respectively. (b) Circadian time-dependent and the mating status dependent expression pattern of *Ac-atrn* in the olfactory tissue of both male and female mosquitoes

8.4 Conclusion

In vertebrates and few invertebrates, a multi-domain proteins *attractin*, facilitate many physiological functions and thus have been regarded as a potential therapeutic target for many neuro-regulatory and sexual disorders. Under multiple innate physiological status of mosquitoes, we evaluated the transcriptional response of *attractin* homolog that was identified from the olfactory system of *An. culicifacies* mosquito. A comprehensive *in silico* analysis and transcriptional regulation studies indicated that *Ac-atrn* not only manages neuro-olfactory associated physiological functions but may also play a crucial role in courtship engagement and other behavioral responses (Figure 8.9).

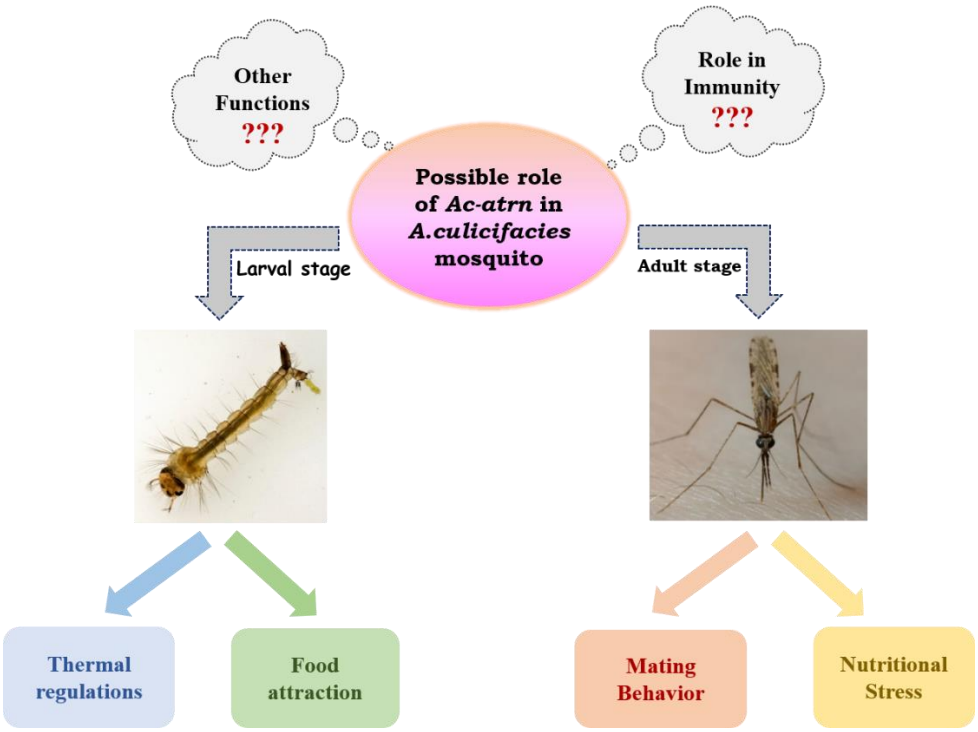

Figure 8.9: Proposed hypothesis for the possible functions of *attractin* gene in the mosquito *An. culicifacies*.

Chapter 9: Conclusion and future implications of the current study

Like other insects, every behavior of mosquitoes' lifecycle is immensely dependent on their ability of odor coding. It is believed that odors mediated responses are predominantly managed by the olfactory encoded Odorant Binding Proteins (OBPs) and Olfactory Receptors (Ors), which influence the mosquito brain to perform a decisive action. Therefore, in the current study we planned to unravel that how the neuro-olfactory systems co-ordinate and regulate blood feeding associated complex behavioral events including host seeking, blood meal uptake, and oviposition in adult female mosquitoes. Using a functional genomics approach, we identified, catalogued and selectively compared the transcriptional responses of neuro-olfactory derived factors, corresponding to physiologically distinct feeding status. We highlight the following key findings that are valuable (i) to understand the blood feeding associated complex neuro-olfactory regulation, and (ii) to identify several unique molecular targets for future functional characterization.

- Our, RNA-Seq study coupled with relative gene expression analysis of the olfactory system of *An. culicifacies* mosquito indicated that blood meal does not cause a global alteration of olfactory gene expression pattern.
- A unique change of the olfactory factors is sufficient to manage distinct mosquito behaviors. Furthermore, a detail transcriptional profiling of the OBPs and ORs genes suggested that a synergistic and concurrent actions of these olfactory factors govern the 'prior and post' blood feeding associated complex behavioral events successfully.
- Our findings also highlight that adult female mosquitoes might take an advantage of the priming effect of the first blood meal exposure, which enables them to learn and adapt for more rapid blood meal uptake during consecutive feeding events.
- Identified a species-specific unique Sensory Appendages Protein (SAP-1 & SAP-2) that may play a crucial role in host-seeking and blood feeding behavior in *An. culicifacies* mosquito and thus act as a novel target to disorient mosquitoes.
- Detail characterization of a novel Ac-quick-to-court *(Ac-qtc)* gene by molecular and behavioral assays indicated that *Ac-qtc* may have dual mode of action in the regulation of a cluster of mosquito olfactory genes that are linked to mating success and/or blood feeding and thus possibly have unique role to manage conflicting demand of mating vs blood feeding in adult female mosquitoes.
- Characterization of another unique gene *Ac-attractin* (*Ac-atrn*) suggested that *Ac-atrn* may have multiple physiological regulatory functions in both aquatic and adult

stages and may also play a crucial role in courtship engagement behavioral response.

- RNA-Seq analysis of the brain tissue of adult female *An. culcifacies* mosquito indicated that a gradual modulation of brain transcripts expression is crucial to manage blood meal associated decision-making events.
- Detail transcriptional profiling of shortlisted brain transcripts engaged in neuro-signaling mechanism suggested that brain is actively engaged to manage metabolic switch associated multi-physiological responses.

In summary, our comprehensive RNA-Seq and extensive transcriptional profiling provide enough evidence that how external stimulus guided olfactory navigation and internal nutritional status guided active brain engagement, together manage and facilitate the successful events of host-seeking, blood feeding and oviposition site finding behavioral response in the adult female mosquitoes (Figure 9.1).

We believe further unraveling the molecular mechanism that how the neuro-olfactory derived key genetic factors regulate each of the crucial behavioral events in adult mosquitoes, may be valuable for future designing of novel disorientation strategy against the deadly mosquitoes.

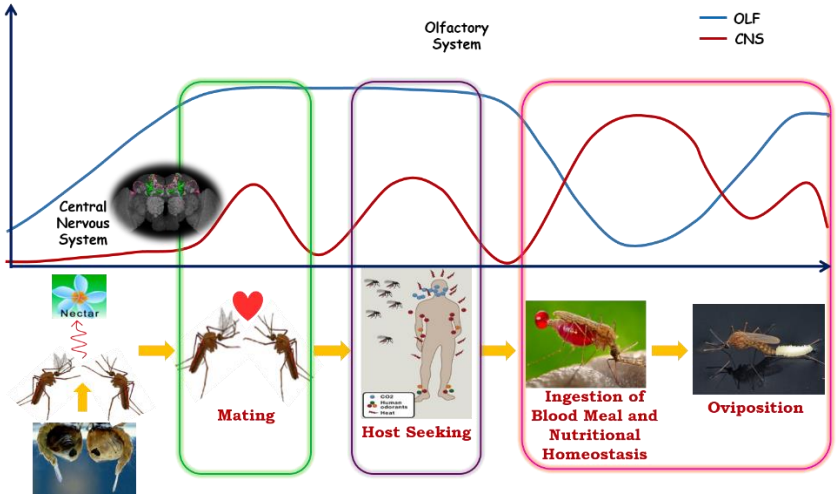

Figure 9.1: A proposed future working hypothesis to find out the molecular relationship of the neuro-olfactory system regulating each life-cycle behavior of mosquitoes. Based on our findings we hypothesize and propose that post-emergence mosquitoes' olfactory system is more active to fulfill the daily requirements. But, a time-dependent activation of the brain may modulate mosquito's decision making abilities controlling distinct behavioral events such as mating, host-seeking, blood feeding, and oviposition.

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A Synergistic Transcriptional Regulation of Olfactory Genes Drives Blood-Feeding Associated Complex Behavioral Responses in the Mosquito *Anopheles culicifacies*

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Decoding the molecular basis of host seeking and blood feeding behavioral evolution/adaptation in the adult female mosquitoes may provide an opportunity to design new molecular strategy to disrupt human-mosquito interactions. Although there is a great progress in the field of mosquito olfaction and chemo-detection, little is known about the sex-specific evolution of the specialized olfactory system of adult female mosquitoes that enables them to drive and manage the complex blood-feeding associated behavioral responses. A comprehensive RNA-Seq analysis of prior and post blood meal olfactory system of *An. culicifacies* mosquito revealed a minor but unique change in the nature and regulation of key olfactory genes that may play a pivotal role in managing diverse behavioral responses. Based on age-dependent transcriptional profiling, we further 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 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 further provide enough evidence that how a synergistic and concurrent action of OBPs-Ors may drive "prior and post blood meal" associated complex behavioral events. A dominant expression of two sensory appendages proteins (SAP-1 & SAP2) in the legs of *An. culicifacies* suggests that this mosquito species may draw an extra advantage of having more sensitive appendages than *An. stephensi*, an urban malarial vector in the Indian subcontinents. Finally, our molecular modeling analysis predicts crucial amino acid residues for future functional characterization of the sensory appendages proteins which may play a central role in regulating multiple behaviors of *An. culicifacies* mosquito.

SIGNIFICANCE

Evolution and adaptation of blood feeding behavior not only favored the reproductive success of adult female mosquitoes but also make them important disease-transmitting

vectors. An environmental exposure after emergence may favor the broadly tuned olfactory system of mosquitoes to drive 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. Our findings suggest that a synergistic action of olfactory factors may govern 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 that "sensory appendages protein" may be a unique target to design disorientation strategy against the mosquito *Anopheles culicifacies*.

Keywords: mosquito, host-seeking, blood feeding, behavior, olfaction

INTRODUCTION

Mosquitoes are one of the deadliest living animals, transmitting a variety of infectious diseases such as malaria, dengue fever, chikungunya and zika fever worldwide. According to WHO report, malaria is one of the major vector-borne diseases that causes 212 million morbidity cases and more than 4 million mortalities (World Health Organization, 2015). WHO recognized that in India, malaria situation is more complex and puts an estimated socio-economic burden of $1.94 billion annually (World Health Organization, 2015). Current tools to control and manage malaria face challenges due to the emergence of drug resistance in parasite and insecticide resistance in mosquitoes (Stein et al., 2009; Petersen et al., 2011; Winzeler and Manary, 2014; Cui et al., 2015; Liu, 2015; Sahu et al., 2015). Thus, alternative molecular tools are required 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 that are able to disorient/alter the adult female mosquitoes host-seeking behavior (Potter, 2014). Therefore, defining the molecular basis of host-seeking behavioral evolution and adaption to blood feeding by the adult female mosquitoes remains central to our understanding. This may probably be due to the complex interaction of genetic and non-genetic factors, driving mosquito navigation (Takken and Verhulst, 2013). In nature mosquitoes encounter many challenges to sustain in daily life viz. they rely immensely on their sense of smell (olfaction) for the majority of their lifecycle stages (Potter, 2014). The well-developed nasal system of mosquitoes is able to detect and discriminate thousands of different odor molecules and thus play an essential role in the facilitation of olfactory guided behavior. 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) (Takken and Knols, 2010). 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 (Takken and Knols, 2010; Carey and Carlson, 2011; Brito et al., 2016). These globular protein molecules showed significant diversity within the same family and are believed to bind with a wide range of hydrophobic odorant molecules. After binding with the odor molecules, OBPs transport it to their respective olfactory receptors present on the olfactory receptor neurons (ORNs) (Takken and Knols, 2010; Fan et al., 2011; Martin et al., 2011). Olfactory receptors (OrX) of insects' are associated with the obligate receptor co-receptor (Orco) on the dendritic membrane of ORN for proper functioning (Takken and Knols, 2010). Orco is not only essential for dendritic trafficking and presentation of the OrX in the membrane but also facilitate the formation of odorant gated ion channels by structural alteration that is opened upon odorant binding (Zwiebel and Takken, 2004; Takken and Knols, 2010).

The genome sequencing of several *Anopheline* sp. facilitates the identification of different olfactory genes including OBPs and Ors from different mosquito species. Functional characterization of few *Anopheline* mosquitoes OBP genes (OBP1, OBP20, OBP7, OBP2, OBP48) highlights their role in host-seeking behavioral activities (Biessmann et al., 2005, 2010; Li et al., 2005; Sengul and Tu, 2008, 2010; Hoffman et al, 2012; Tsitsanou et al., 2013; Ziemba et al., 2013). Consequently, de-orphanization of several odorant receptors (AgOr1, AgOr2, AgOr8, AgOr5, AgOr65) from *An. gambiae* also showed their specificity to human-specific odorant molecules (Hallem et al., 2004; Carey et al., 2010). After binding of the odorant molecules with their cognate receptors, the actual signal transduction cascade is initiated which involves either the activation of ligand-gated ion channels or stimulation of the secondary messenger pathway (Takken and Knols, 2010). In insects, including mosquitoes, a combinatorial coding mechanism of the olfactory system is believed to increase the sensitivity of the odorant reception, which enables them to respond to specific odorants (Martin et al., 2011; Andersson et al., 2015). 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 needs to 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 2 min (Zwiebel and Takken, 2004; Benoit et al., 2011; Sim et al., 2012; McMeniman et al., 2014; Cardé, 2015; Van Breugel et al., 2015; Won Jung et al., 2015). Just after the piercing organ (proboscis), it is the salivary gland which mediates 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 (Sharma et al., 2015b), but the molecular nature of the olfactory and neuro-system in regulating the salivary gland function is yet to unravel.

Mosquitoes after taking a blood meal, need to enter into a new habitat favoring successful oviposition (Rinker et al., 2013; Day, 2016). In fact, after blood meal acquisition, mosquitoes undergo two major behavioral switching events; (i) searching for suitable site(s) for temporary resting and completion of blood meal digestion (∼30 h) which is necessary for egg maturation (48–72 h); and (ii) finding a proper oviposition site for successful egg laying (Taparia et al., 2017). After completion of egg laying event, the adult female mosquitoes regain their host-seeking activity for a second blood meal to complete the next gonotrophic cycle (Takken et al., 2001; Rinker et al., 2013). Notably, "prior and post" blood meal associated habitats may have a significant difference in their physical, chemical and biological characteristics (Day, 2016), but the molecular basis that how olfactory-driven factors manage these complex events is still not well understood (Chen et al., 2017).

Immediately after mosquito emergence, an exposure to diverse environmental/chemical cues facilitate the maturation and learning of the olfactory machinery components (sensory appendages, maxillary palps and proboscis) to govern common innate behavioral activities such as nectar sugar feeding and mating in both the sexes (Takken and Verhulst, 2013; Lutz et al., 2017). However, it is yet not clear whether the mating events have any direct impact on the initiation of host seeking and blood feeding behavioral responses. Our recent finding suggested that *quick-to-court* protein may have a crucial role to meet the conflicting demand of sexual mate partner finding and/or a suitable vertebrate host finding by regulating the expression of unknown olfactory genes in adult *An. culicifacies* mosquito (De et al., 2017). In fact, the organization of the olfactory components is morphologically similar in both the sexes but carries unique structural differences which are responsible for discrete temporal peaks of activities to sense swarm and identify sex partner for a successful mating event (Pitts et al., 2011). However, in case of adult female mosquitoes, we opined that the evolutionary forces might have driven an extra specialization of the olfactory components such as proboscis, enabling rapid host seeking and blood feeding behavioral adaptation. In other words, we termed this highly sex-specific extra specialization as an "evolutionary speciality" which not only evolve adult female mosquitoes as a fast blood feeder but make them a potent vector for many disease pathogens. 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 perception 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 RNA-Seq analysis of the complete olfactory system of adult female *An. culicifacies* mosquito, a dominant Indian malarial vector. A comprehensive molecular and functional annotation of RNA-Seq data unraveled 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 for future functional validation and characterization as a unique target to design disorientation strategy against the mosquito *An. culicifacies*, responsible for more than 65% malaria cases in India (Sharma and Dev, 2015).

MATERIALS AND METHODS

Figure 1 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 (Thomas et al., 2014; Sharma et al., 2015b). 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 to 1 day of age, 30 min post blood fed and 30 h post blood fed *An. culicifacies* mosquito and collected in Trizol Reagent. Total RNA isolated from the collected olfactory tissues of approximately 30 mosquitoes was pooled to form one single sample and a double-stranded cDNA library for each set of naïve, 30 min and 30 h post blood meal, was prepared by a well-established PCR-based protocol described previously (Dixit et al., 2011; Sharma et al., 2015b). Whole transcriptome sequencing of the olfactory tissue was performed using the Illumina MiSeq 2 X 150 paired-end library preparation protocol. The sequencing data analysis pipeline is shown in **Figure 1**. Briefly, raw reads from each set were processed for removing the adaptors and low-quality bases (<20). A denovo clustering using CLC Genomics Workbench (V6.2) (Zhu et al., 2014) was used to build final contigs/transcripts dataset with default parameters (contig length ≥ 200, Automatic word size: Yes, Perform Scaffolding: Yes, Mismatch cost: 2, Inserstion cost: 3, Deletion cost: 3, length fraction: 0.5, Similarity fraction: 0.8). Finally, assembled transcriptome was used for CDS prediction and annotation using transdecoder and BLASTX at *e*-value $1e^{-6}$, respectively.

For a comprehensive differential gene expression (DGE) analysis we used DESeq R Package as described earlier (Chen

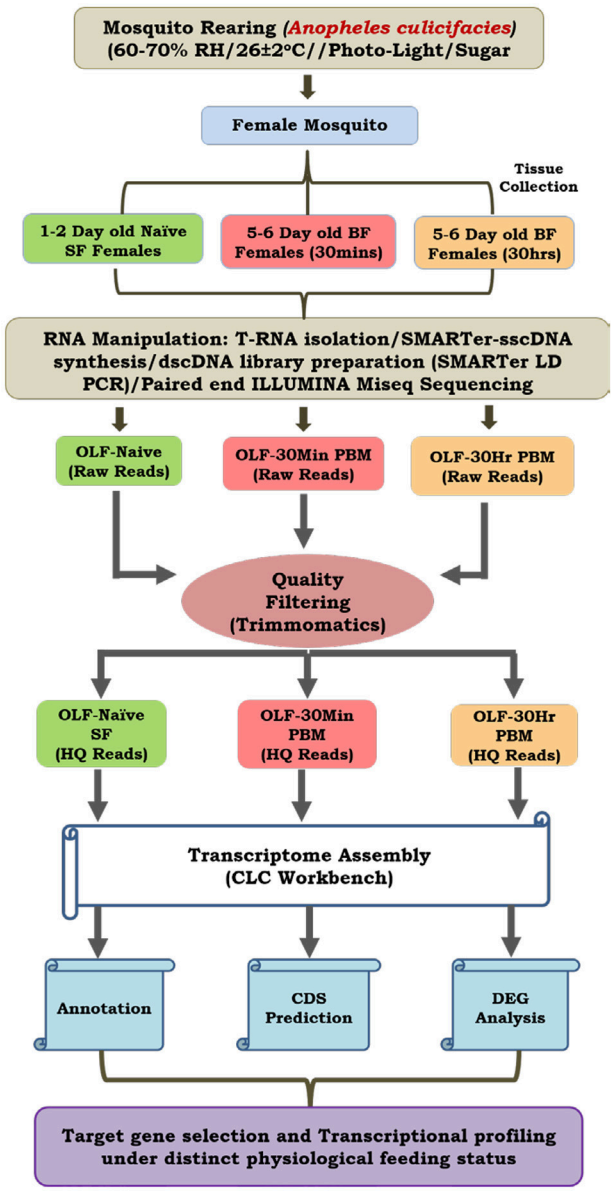

FIGURE 1 | A technical overview to decode the hard-wired genetic structure of the olfactory system of *Anopheles culicifacies*. We sequenced and analyzed three RNA samples extracted from the olfactory tissues of approximately 30 mosquitoes individually and pooled to form one single sample. With this strategy, we were able to quantify and validate the estimation of gene expression, expected with a minimum chance of aberrations as described earlier (Sharma et al., 2015b).

et al., 2017). Briefly, the high quality reads for each sample were mapped on their respective set of CDS/transcripts and FPKM (Fragments Per Kilobase of Exon Per Million Fragments Mapped) values were calculated using following formula i.e., FPKM = 10^9 x C / (N x L), where C is the number of reads mapped onto the CDS; N the total number of mapped reads in the experiment; and L is the number of base pairs in the CDS. The common hit accessions based on BLAST against NR database were identified for differential gene expression analysis. CDS were further classified as up and down-regulated based on their log fold change (FC) value, which was calculated by the using the formula: FC = Log_2 (Treated/Control). Because, DESeq calculates raw *p*-values using a negative binomial distribution accounting technical and biological variables, and later *p*-values are corrected for multiple testing using the Benjamini-Hochberg statistical procedure which controls false discovery rate (FDR). Transcripts pairs whose read numbers displayed a greater than two-fold difference with $P < 0.05$ was listed as differentially expressed genes.

Identification and Molecular Cataloging 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 OBPs database of mosquito and *Drosophila* was re-queried against *An. culicifacies* draft genome/predicted transcripts databases available at www.vectorbase.org and build up the final OBP catalog for phylogenetic analysis as detailed in the Figure S1. A PDB database homology search analysis and GO annotation was used to identify and catalog 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 h 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 (Rund et al., 2013). At the same time other tissues such as. 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 also 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 s followed by 55°C for 15 s and again 95°C for 15 s were completed before deriving a melting curve. Each experiment was performed in three independent biological replicates to better evaluate the relative expression. Actin or S7 gene was used as internal control in all the experiment and the relative quantification was analyzed by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Differential gene expression was statistically analyzed using student *t*-test.

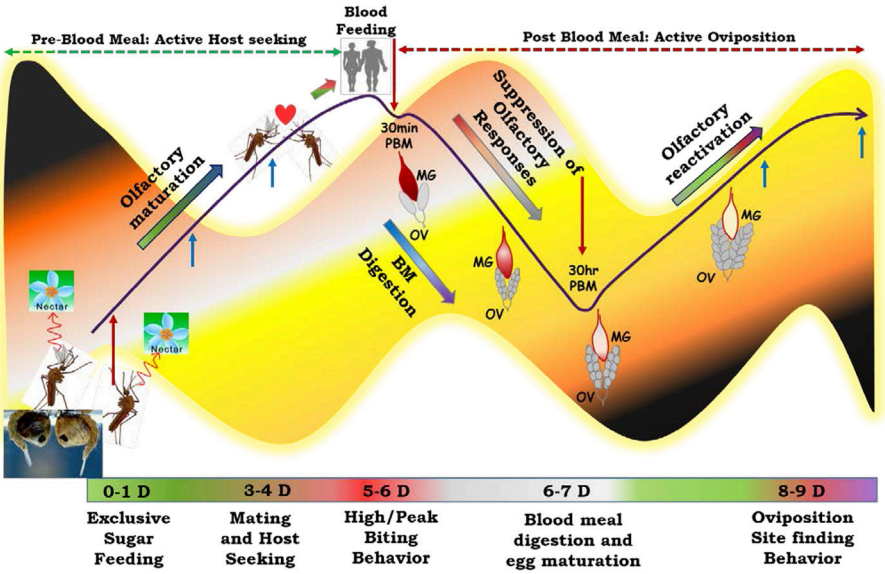

FIGURE 2 | Working Hypothesis to establish functional co-relation of the olfactory system under distinct feeding status: Adult mosquitoes, just after emergence from pupae are exclusive sugar feeders and dependent on nectar sugar to acquire energy for flight activity. Exposure of the adult mosquitoes to the diverse aromatic environment facilitates their learning and maturation of the olfactory system which enables 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 30 h 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 h of blood feeding the blood almost digested in the midgut and maturation of egg reached a threshold level which reinforces the mosquito to perform to next level of behavior. Thus, recovery/reactivation of the olfactory responses occurs to find a suitable site for egg laying/oviposition. To capture this molecular snapshot and track the events, we collected olfactory tissues at three different physiological conditions for RNA-Seq 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).

Blood Meal Time Series Follow Up

Figure S9 represents a technical overview of the blood meal follow up experimental protocol. Briefly, the olfactory tissues were collected from 25 to 30 adult female mosquitoes for both naïve sugar-fed and blood fed mosquitoes at different time points. Olfactory tissues collections were initiated from 0 to 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 min 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 30 h and 72 h post blood fed the gravid females were kept for oviposition and again dissected OLF tissues after 24 h of the egg laying event. Second blood meal was provided to the egg laid mosquitoes and final collection of OLF tissues was done after 30 h of 2nd blood meal. Initially, relative expression data were interpreted to evaluate a general response using one way analysis of variance (ANOVA) for multiple comparison, however, wherever required "test" sample data was compared with "control" data set and statistically analyzed using Student's *t*-test.

Structural Modeling of SAP1 and SAP2

The structure prediction analysis of SAP1 and SAP2 proteins from *An. culicifacies* was carried out through searching of a template for each query proteins against PDB database using BLASTP algorithm. Based on highest query coverage, identity and *e*-value, 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 using DOPE (Discrete Optimized Protein Energy) score, which is favored by the lowest cumulative energy score for the whole structured model. The selected model was further validated by Ramachandran plot using PROCHECK software which estimates the stereo-chemical quality of the residues in allowed, disallowed and favorable regions. Finally, the selected models were used for binding site prediction using COACH software.

RESULTS

Blood Meal Causes Modest but Unique Changes to Olfactory Responses

To decode and establish the possible molecular relationship managing "prior and post" blood meal behavioral events we developed a working hypothesis (**Figure 2**), a plausible mechanism which may have a significant influence on mosquito feeding and survival in diverse ecologies. To test this hypothesis, first we generated and analyzed a total of ∼122 million RNA-Seq reads of the olfactory tissues collected from 1 to 2 day old

TABLE 1 | Catalogue of Odorant Binding Proteins of *An. culicifacies*.

Sl no.	Sample name	Number of OBPs transcripts
(A)		
1.	Ac-OLF-Naïve	14
2.	Ac-OLF-30 min PBM	10
3.	Ac-OLF-30 h PBM	12
4.	Ac-genome retrieved	27
	Total OBPs in *An. culicifacies*	63
Sl no.	**Family of OBPs**	**Number**
(B)		
1.	Classic OBPs	26
2.	Plus-C OBPs	13
3.	Two-domain OBPs	13
4.	Other chemosensory proteins	11

naive (Nv), 5- 6-day old immediate blood fed (30 m-2 h PBM) and 30 h post blood fed (30 h PBM) mosquitoes (**Table 1**). We chose 30 h PBM as a critical time when completion of blood meal digestion occurs in the midgut, which may have a direct influence on the reactivation of the olfactory system (Figure S2) (Gonalves et al., 2009; Rinker et al., 2013; Taparia et al., 2017). For molecular and functional annotation, we assembled each transcriptomic database into contigs/transcripts and compared against multiple molecular databases as described earlier (Sharma et al., 2015b). Supplementary Table 1 represents details of the annotation kinetics of mosquito olfactory databases.

To test whether blood meal alters the global expression pattern of the olfactory transcriptome, we performed a differential gene expression analysis. Initial attempt of mapping cleaned reads to the available draft reference genome failed to yield quality results, probably due to poor annotation (Figure S3). Alternatively, we mapped all the high quality reads against *denovo* assembled reference map, as described earlier (Sharma et al., 2015b). Blood meal causes a modest shift in the transcriptome expression (**Figure 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* (Rinker et al., 2013).

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 (Supplementary Table 2 and Dataset S1). As expected, ∼10% transcripts expression was further reduced in 30h post blood fed olfactory tissue samples while only 2% transcripts were up-regulated when compared to naive sugar-fed mosquitoes (Supplementary Table 2). Interestingly, a comprehensive annotation analysis also predicted that basic composition of the mosquito olfactory tissue does not alter significantly (**Figures 3B–D**). This observation allowed us to further 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 signaling in the blood fed adult female mosquitoes (**Figures 3B–D**). To clarify this complexity, we manually shortlisted the olfactory transcripts either based on their FPKM abundance and/or predicted coding nature and analyzed 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 toward 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 cataloged a total of sixty-three OBP genes by homology search analysis 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 Supplementary Table 3), as described earlier for the mosquito *An. gambiae* (Manoharan et al., 2013).

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 (Figure S4A). Whereas, Plus-C OBPs and more dominantly Atypical OBPs seem to be unique to the mosquitoes suggesting their possible involvement in the evolution and adaptation of blood feeding behavior of adult female mosquitoes (Figures S4B,C).

Interestingly, differential gene expression (DGE) data indicated that blood meal restricted the expression of common OBP transcripts (Figure 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 change in the feeding status i.e., naive sugar to blood feeding. To further validate and unravel this unique relationship of OBPs regulation, we examined the RT-PCR based expression of at least 11 putative OBP transcripts under distinct feeding status of the mosquitoes. In this analysis, we also included two chemosensory proteins (CSPs) named sensory appendage protein (SAP1 & SAP2) having a dominant expression in the naive mosquito olfactory tissue (Supplementary Table 3).

Our Zeitgeber time scale expression showed that out of tested nine OBPs transcripts, at least 6 OBP transcripts showed a >2-fold modulation in their expression during late evening to midnight, in the 6-day old naïve mosquitoes (**Figure 4A**). These data also corroborate with the previous observation that the natural active biting behavior of *An. culicifacies* mosquito occurs in the mid-night (Singh et al., 1995; Basseri et al., 2012). 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. Apparently, we also observed a transient suppression (30 min)

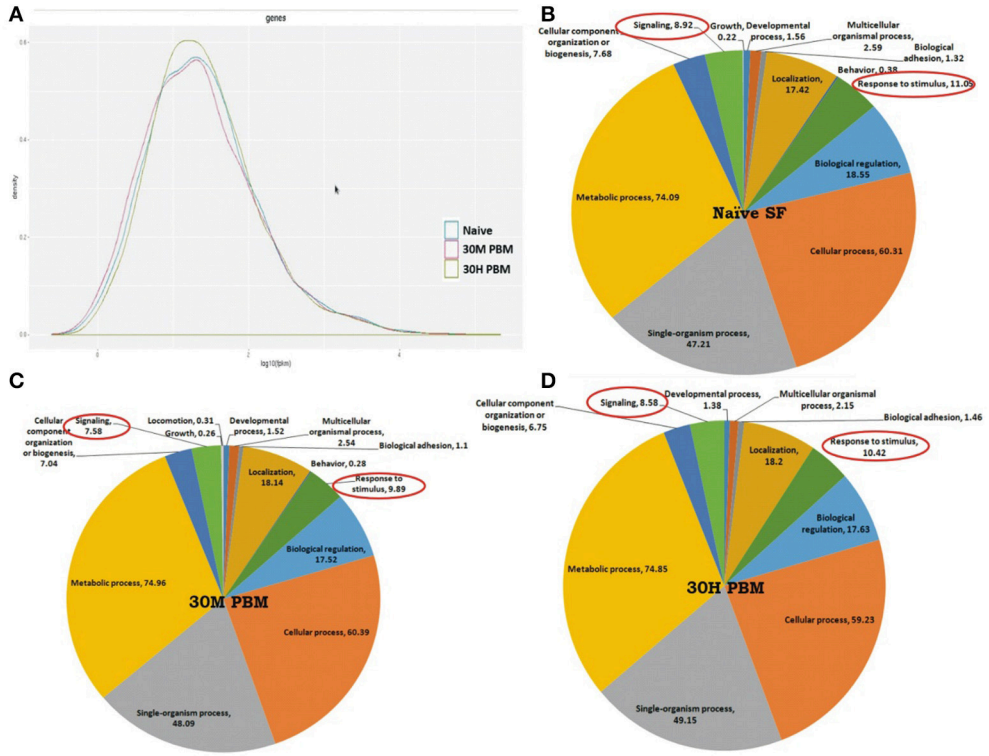

FIGURE 3 | Blood meal cause modest changes in the molecular architecture of the mosquito olfactory system**: (A)** Read density map of the compared Naive; 30 m and 30 h post blood meal (PBM) transcriptomic data of olfactory system; **(B–D)** Functional annotation and molecular catalog of olfactory transcriptome (Biological Process/Level2/% Transcripts). The red circle marks the genes selected for transcriptional response monitoring. (See Text).

and rapid recovery of OBPs expression just after a first blood meal (**Figure 4B**). However, surprisingly, OBP7 showed a unique pattern of a consistent up-regulation till the 6th day when compared to a gradual enrichment of other tested OBP and SAP after 3-day post-emergence in the naive adult female mosquitoes. However, it is yet to be clarified whether an early enrichment of OBP7 has any important role in aging mosquitoes' olfactory responses.

Innate Physiological Status May Influence Olfactory Receptor Responses to Manage Behavioral Switching Events

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 cataloged a total of 603 unique transcripts linked to response to stimulus and signaling (RTSS) categories (**Figures 3B–D**), encoding diverse nature of proteins such as anion binding, nucleic acid binding, receptor activity, hydrolases and transferase activity (**Figure 5A**). A comparative GO score distribution analysis predicted lower score hits for the blood-fed cohorts than naive mosquitoes (**Figure 5A**). Surprisingly, out of 603 transcripts, we noticed only 110 transcripts were common to all, while >100 transcripts remain uniquely associated with individual physiological conditions compared in the study (**Figure 5B**).

Olfactory receptors play a central role to receive and communicate the initial chemical message to the higher brain center through ORNs for decision-making events. Thus, we made a catalog of 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 (Supplementary Table 4). Interestingly, a cluster of 19 different olfactory receptor genes was found to be expressed abundantly and exclusively in the naïve mosquito (Supplementary Table 4). 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 (Supplementary Table 4). Observation of the constitutive expression of Orco and few other Ors and Grs (totaling 10 transcripts) in all the experimental conditions highlighted the importance of Orco for the presentation of other receptors in the olfactory system.

Unlike OBPs, poor modulation of olfactory receptor gene expression under circadian rhythm (**Figure 5C**) suggested their minimal role 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

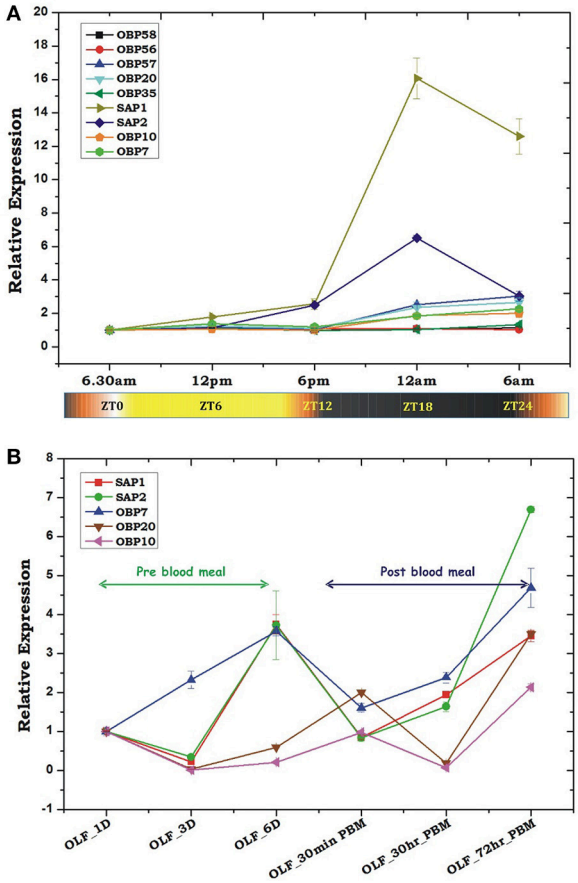

FIGURE 4 | Transcriptional profiling of the odorant-binding protein genes (OBPs) under different circumstances. **(A)** Rhythmic expression of OBP genes in the adult female's olfactory tissues (OLF) according to different zeitgeber time (ZT) scale, where ZT0 indicate the end of dawn transition, ZT11 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 1, 3, and 6 day old sugar-fed mosquitoes which were then provided blood meal and then the olfactory tissues were collected after 30 min of post blood fed and 30 and 72 h of post blood-fed mosquitoes. The significance of suppression of OBP genes expression after 30 h of post blood meal are as follows: SAP $\leq$ 0.004; SAP2 $\leq$ 0.039; OBP7 $\leq$ 0.007; OBP20 $\leq$ 0.0004; OBP10 $\leq$ 0.003.

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 experiment. 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 (**Figure 5D**).

First blood meal to the 6th-day old naïve mosquitoes initiates the suppression of almost all the olfactory receptor transcripts within 30 min of blood feeding, whose expression almost ceased to a basal level at 30 h post blood meal, except the slight up-regulation of two transcripts named Or42 and Or62 (**Figure 5D**). Apparently after 30 h PBM, we observed a significant modulation of the receptor gene expression which started enriching till 72 h 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 (**Figure 5D**).

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. 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 (**Figure 6A**). Though, the involvement of G-proteins and related metabotropic signaling mechanism in the olfactory signal transduction of insects remain controversial, however, our observation of a rapid and consistent induction of adenylyl cyclase gene after 30 m PBM (**Figure 6B**), supported the previous hypothesis that the synthesis of the secondary messenger, cAMP by adenylate cyclase, facilitates odorant mediated signal transduction process which further influence downstream behavioral responses (Takken and Knols, 2010). Surprisingly, finding of <1% of transcripts encoding putative immune proteins suggested that the maintenance of a basal level of sterility is essential for proper olfactory functions (Figure S6).

Sensory Appendages Proteins as a Unique Target to *Anopheles culicifacies*

To test whether any species-specific olfactory derived genetic factors have any differential regulation likely to influence the behavioral responses, we compared the expression of at least 6 OBPs transcripts between two laboratories reared mosquito species *An. stephensi* and *An. culicifacies*. Though, both are dominant malaria vectors in urban and rural India, respectively, but display a significant difference in their behavioral properties such as feeding, mating, biting preferences etc., (personal observation/ST-S5). In this analysis, we also included two SAP proteins, which showed a high induction than other OBPs in the olfactory system of *An. culicifacies* mosquito at midnight (**Figure 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 *An. culicifacies* mosquito (**Figures 7A,B**). Together these data indicated that *An. culicifacies* may draw an extra advantage of having more sensitive appendages, possibly to favor more active late night foraging behavior, than *An. stephensi*. Though,

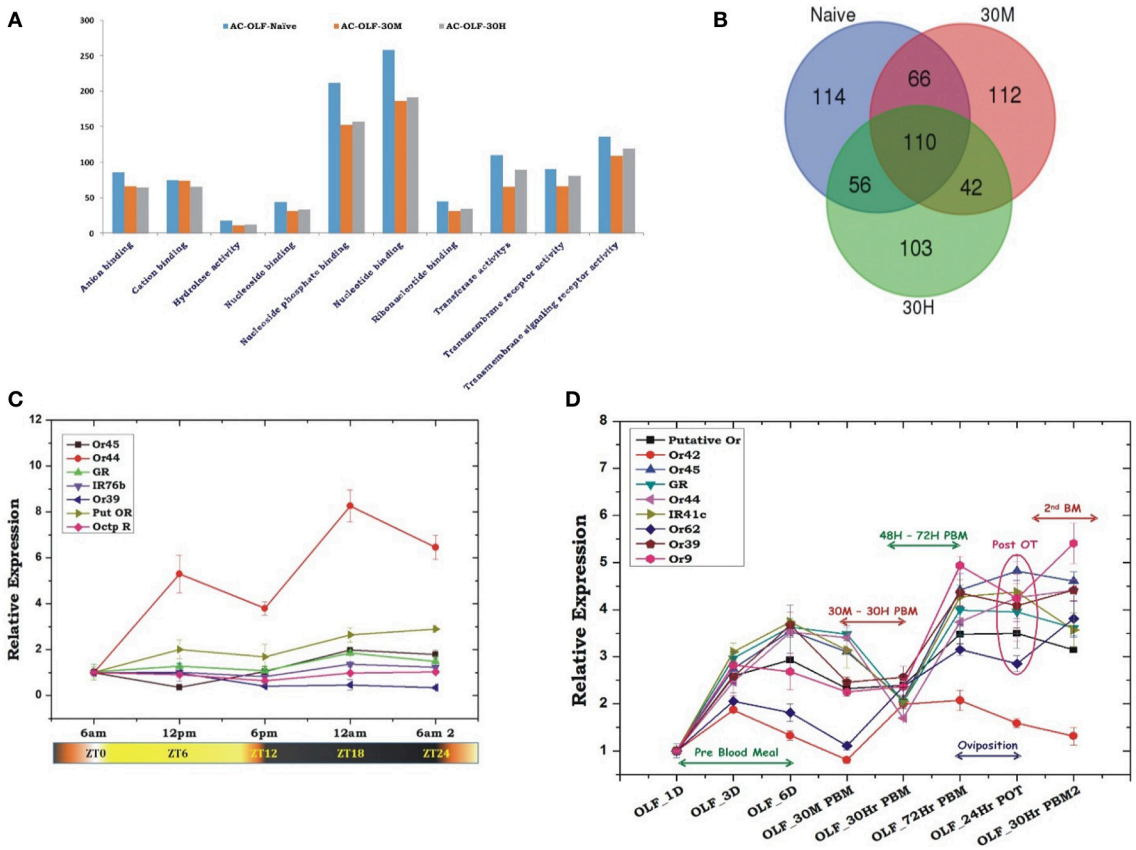

FIGURE 5 | Blood meal modulates odorant receptors expression. **(A)** A comparative GO score distribution analysis of the response to stimulus and signaling transcripts of naïve and blood-fed mosquitoes. **(B)** Venn diagram showing common and unique transcripts of response to stimulus and signaling 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 a different time point after blood feeding, viz. OLF-30 M: 30 min post blood fed (PBM); 30 h-PBM: 30 h of PBM; 72 h-PBM: 72 h of post blood meal; then the mosquitoes were kept for oviposition (egg laying), and again the olfactory tissues were collected 24 h of post oviposition (24 h-POT). Finally, the 2nd blood meals were provided to the egg laid mosquitoes and collected olfactory tissue 30 h of second blood meal (30 h-PBM2). A multiple comparison analysis (ANOVA) revealed a significant modulation in the expression of each gene (mean significant $p < 0.05$). Further, the significance of suppression of OR genes expression after 30 h 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.

defining the basis of host preference remains uncertain, because *Anopheline* mosquitoes have opportunistic feeding behavior which is largely influenced by nature of the host availability (Thiemann et al., 2011). A close association of Ac-SAP proteins with the anthropophilic *Anopheline* mosquitoes (**Figure 7C**, Figure S7A), 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*.

The above findings further prompted us to carry out a 3D structure modeling 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 the reference SAP protein, we applied a template based comparative molecular modeling 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%, favoring their suitability for structure prediction.

TABLE 2 | Number of Odorant Receptors retrieved from the olfactory tissue of naive and blood fed *An. culicifacies* mosquito.

Sl. no.	Sample name	Number of olfactory receptors transcripts
1.	Ac-OLF-naive	32
2.	Ac-OLF-30 min PBM	11
3.	Ac-OLF-30 h-PBM	7
4.	Total	50

Out of the 50 modeled 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 (**Figure 7D**, Figure S7B).

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

(**Figure 7E**, Figure S7C). 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 (**Figure 7F**, Figure S7D). 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 (**Figure 7G**, Figure S7E).

In our analysis, we observed the presence of at least two conserved cysteines (CYS52 and CYS55) residues in the loop region of SAP1 and SAP2 proteins, which may likely have involved in di-sulfide 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.

DISCUSSION

It is well known that a circadian dependent modulation of olfactory responses significantly influences the complex behavioral responses in both sexes of *Anopheline* mosquito species (Rund et al., 2013). However, the evolution of the more specialized olfactory system of adult female mosquitoes favored their unique adaptation to blood feeding behavior. The female olfactory system comprises of three olfactory appendages i.e., (a) the antennae, (b) the maxillary palps and (c) female specific proboscis, which may encode a variable number of factors responsible for maintaining female specific daily olfaction rhythms such as host seeking, blood feeding, and oviposition behavior. Since, peripheral antennal appendages harbor more diverse OBPs, Ors, and other factors, it acts as the principle chemosensory organ that respond to wide range of volatile odors. Therefore, major electrophysiological and molecular studies have been focussed on this olfactory component. A few recent studies examining global profile change in response to daily rhythms and blood feeding highlighted the important role of the antennal transcripts/proteins in the modulation of distinct behavioral responses of *Anopheline* mosquitoes (Rinker et al., 2013; Rund et al., 2013; Chen et al., 2017). While the maxillary palps encode unique receptor proteins such as Or8, Or28, and Orco, which respond to carbon dioxide to enable mosquitoes for a successful navigation toward vertebrate hosts (Pitts et al., 2011). In the close vicinity of the targeted hosts, female mosquito's proboscis encoded factors rapidly engaged to complete the blood meal uptake process in less than 2 min. The molecular basis that how olfactory appendages encoded factors interlinked with each other to drive highly sex-specific pre-and post-blood meal behavioral events are not well understood yet.

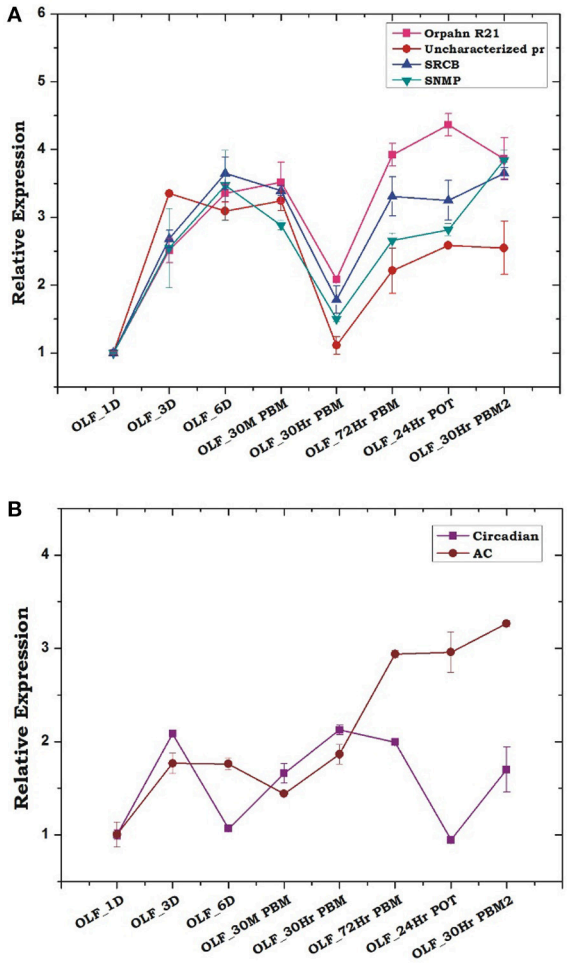

FIGURE 6 | Transcriptional responses of other olfactory genes hypothesized to play a crucial role in host-seeking and blood-feeding behavior. **(A)** Relative expression profiling of other receptor genes according to blood meal time series (described in **Figure 5**). Orphan R21: Orphan receptor 21; Uncharacterized Pr, Uncharacterized protein; SRCB, Scavenger Receptor class B; SNMP, Sensory neuron membrane protein. **(B)** Transcriptional profiling of other signaling 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 30 h of post blood meal are as follows: Orphan R21 $\leq$ 0.002; Uncharacterized pr $\leq$ 0.002; SRCB $\leq$ 0.006; SNMP $\leq$ 0.007.

We have recently demonstrated that adult female mosquitoes are evolved with the unique ability of salivary gland gene expression switching to manage meal specific "prior and post" blood meal responses (Sharma et al., 2015b). Here, we further extended this idea to decode and trace the possible molecular link that how the olfactory factors of adult female *An. culicifacies* mosquitoes drive sex-specific host-seeking, blood-feeding and oviposition behavior. To establish the plausible mechanism of the olfactory system, we developed a working hypothesis (**Figure 2**) and compared the transcriptional response of the olfactory derived transcripts, modulating in responses to changes in the

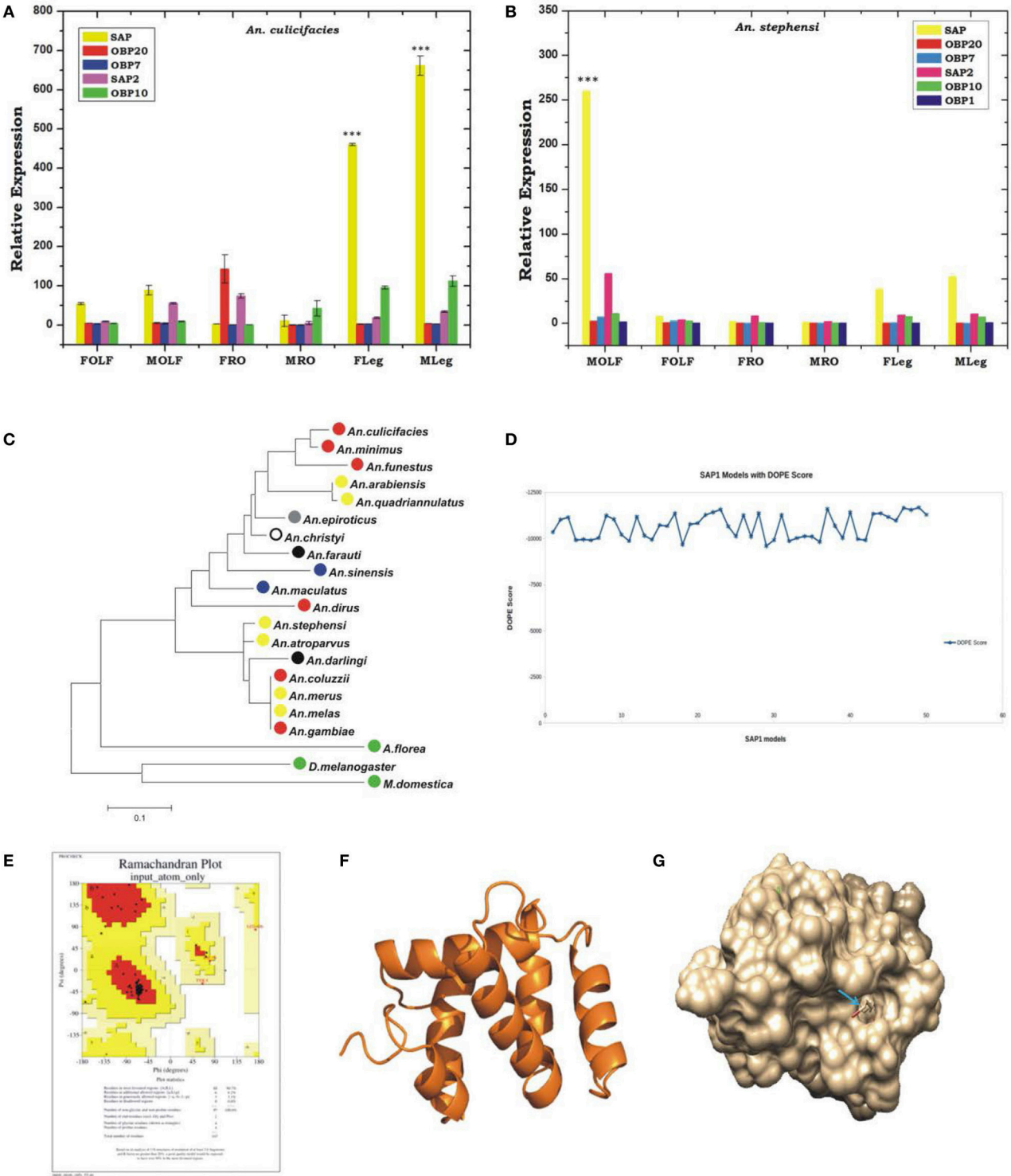

FIGURE 7 | Comparative transcriptional responses of Odorant binding protein genes between two major Indian vectors and structural characterization of one of the potent OBP gene. **(A,B)** Sex and tissue-specific relative expression profiling of OBP genes in *An. culicifacies* **(A)** and *An. stephensi* **(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. Color-coded circle represents the nature of the mosquitoes host preferences e.g., Red circle, Strongly Anthropophilic; Blue circle, Strongly Zoophilic; Yellow circle, Opportunistic Anthropohilic and/or Zoophilic; Black circle, moderate Anthropophilic; Gray circle, Moderate zoophilic; Blank Circle, Unknown; Green circle, non-blood feeder. **(D)** DOPE score analysis for SAP1. **(E)** Ramachandran Plot of SAP1 protein. **(F)** 3-dimensional protein structure of the Ac-SAP1 protein. **(G)** The binding site of SAP1 protein showed in space fill with nearby residues in stick form. ***$p \leq 0.0001$.

feeding status. Surprisingly, an observation of a limited change in the global response of the olfactory system of *An. culicifacies* mosquito partly corroborates with the similar changes in the limited pool of antennal chemosensory genes in *An. gambiae* (Rinker et al., 2013). Taking in account of the nature of tissues i.e., the selected peripheral sensory appendages investigated in previous studies, 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. To unravel the molecular nature and function of the olfactory factors, we annotated, cataloged and selectively profiled the expression of OBPs, Ors and other members of chemosensory genes.

An initial comparison of the annotated transcripts revealed that first blood meal not only delimits the transcripts numbers but also enriches the expression of many unique transcripts having similar functions. Once reached to its saturation level, the expression of selected olfactory transcripts did not alter significantly, when offered an un-interrupted sugar meal to the aging mosquitoes (Figures S8A,B). 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 fed to blood fed status. Furthermore, a zeitgeber time scale experiments suggested that midnight hyper activities of OBPs, especially sensory appendages proteins (SAP-1 and SAP-2), are able to drive female specific host-seeking behavioral activities of naive adult female *An. culicifacies* mosquitoes, supporting the previous finding in other *Anopheline* mosquito species (Biessmann et al., 2005; Iovinella et al., 2013).

Our observation of a transient change in the expression of selected OBP transcripts, 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. 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 (Wong et al., 2011; Phasomkusolsil et al., 2013; Rinker et al., 2013; Lindh et al., 2015). 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 (Carey and Carlson, 2011; Martin et al., 2011; Andersson et al., 2015). Though previous studies suggested that first blood meal causes the alteration of OBP/Ors mediated odor sensitivity (Rinker et al., 2013), how olfactory receptors superintend and co-ordinate between innate and primed/adaptive odor responses remains largely unknown (Lutz et al., 2017). We hypothesize that a harmonious action of OBPs and Ors, which are involved in downstream odorant signal transduction cascade, may have significant influence on behavioral switching events.

To test this hypothesis, we profiled the expression of selected Ors transcripts in response to two consecutive blood meal follow up experiment, which included at least one gonotrophic cycle completion. Supporting the previous reports, we also observed that a first blood meal initiated a gradual suppression of all the olfactory receptor transcripts within 30 min of blood feeding, which was further ceased to the basal level at 30 h post blood meal. However, surprisingly, we observed a two-fold up regulation of all the receptor transcripts in response to second blood meal, when compared the expression after 30 h of first blood meal. Together, these data 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.

Two most potent Indian malarial vector species *An. culicifacies and An. stephensi* have been reported to show predominantly zoophilic and anthropophilic behavior, respectively (Joshi et al., 1988; World Health Organization, 2007; Sharma and Dev, 2015), but the molecular basis of such biological variation is yet to unravel. Emerging evidence suggested that a significant genetic difference exists among various *Anopheline* mosquito species, including *An. stephensi* and *An. culicifacies* (Dash et al., 2007; Sharma et al., 2015a). 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, SAP have been identified and characterized as a key molecular target in many *Anopheline* mosquitoes involved in host-seeking behavior (Biessmann et al., 2010; Sengul and Tu, 2010; Ziemba et al., 2013), but remains poorly understood in Indian vectors.

Therefore, to test whether species-specific olfactory derived genetic factors have any differential regulation, we compared the tissue expression of selected OBP transcripts between two laboratories reared mosquito species i.e., *An. stephensi* and *An. culicifacies*. Surprisingly, a higher expression of SAP-1 and SAP-2 in the legs of mosquito *An. culicifacies* indicated that this mosquito species may have drawn an extra advantage of having more sensitive appendages, possibly to favor more active late night foraging behavior than *An. stephensi*. A 3D molecular modeling analysis not only predicted the presence of at least two conserved cysteines (CYS52 and CYS55) residues in the loop region of SAP1 and SAP2 proteins but also suggested that binding pocket may form a tunnel-like structure, preferred by long aliphatic molecules. While the presence of conserved negatively charged aspartic acid and polar tyrosine at one end of binding pocket suggested their role in ligand binding. Though previously SAP has also been identified from other *Anopheline* mosquito species but their role in host-seeking and blood feeding behavior remains poorly understood (Biessmann et al., 2005; Iovinella et al., 2013). Encouraged by the above observation, we selected SAP as a unique target that may be crucial to design an effective disorientation strategy against *An. culicifacies* mosquito, an important malaria vector in rural India.

CONCLUSION

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

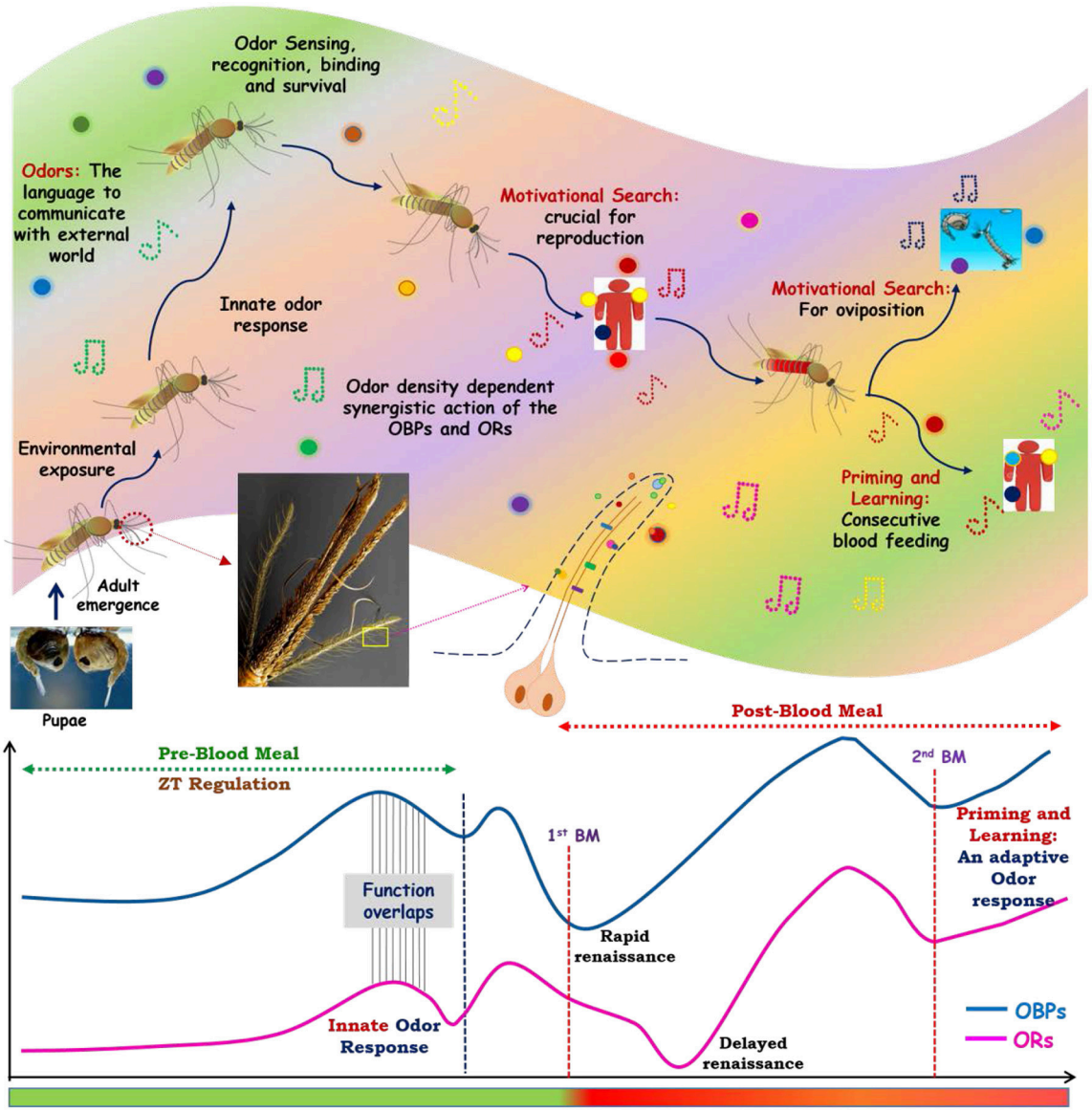

FIGURE 8 | How smart actions of olfactory system manages blood feeding associated odor response: an evolutionary speciality of adult female mosquitoes. After emergence from pupae adult mosquitoes are exposed to the overwhelmed odor world, where odorants chemicals act as a language of communication with the external world. The sophisticated innate olfactory system of mosquitoes enables them to recognize and differentiate this 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 toward 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 to vertebrates facilitates 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.

successful "prior and post" blood feeding associated behavioral complexities. A comprehensive RNA-Seq and extensive transcriptional profiling data, further strengthen the hypothesis 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. However, an extended blood meal follows up experimental data analysis further 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 take 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 (**Figure 8**).

In summary, we decoded and established a possible functional correlation that how coherent and smart actions of olfactory encoded factors enabled adult female mosquitoes to meet and manage the blood feeding associated complex behavioral activities (**Figure 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.

DATA DEPOSITION

The sequencing data were deposited to National Center for Biotechnology Information (NCBI) Sequence Reads Archive (SRA) system (BioProject accessions: PRJNA414162; BioSample accessions: SAMN07981002, SAMN07972755, and SAMN07775994).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: TD, RD; Performed the experiments: TD, TT, SV, DS, VS, PS, CC, SK, ST, JR; Analyzed the data: TD, RD; YH; Contributed reagents, materials, analysis tools: YH, RD, KP; Wrote the paper: TD, RD, YH, KP.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00577/full#supplementary-material

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Transcriptional responses of *attractin* gene in the mosquito *Anopheles culicifacies*: A synergistic neuro-olfactory regulation

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ABSTRACT

Background & objectives: Attractin, is a large multi-domain protein which has regulatory functions in multiple physiological processes and thus have strong therapeutic potential. In invertebrates, it was first identified as a water-borne protein pheromone that plays important role in chemical communication and coordinates reproductive activities. But its role in mosquitoes/insects remains unknown. Our unexpected discovery of *attractin* homolog from the olfactory tissue of *Anopheles culicifacies* mosquito prompted us to investigate the possible role of *Ac-attractin* (*Ac-atrn*) in diverse behavioural responses *e.g*. feeding, mating and other non-genetic stresses.

Methods: A homology search analysis was performed to identify the full length *attractin* (*Ac-atrn*) gene of *Anopheles culicifacies* mosquito. To unravel its molecular function during external and internal stresses, extensive Real-Time PCR was performed in the neuro-olfactory tissue of adult mosquitoes as well as in the larval stages. Further, a behavioral assay was conducted to elucidate its role in mosquitoes mating behavior.

Results: The results indicated that *Ac-atrn* is a 3942 bp long transcript which encodes a 1313 amino acid protein, having multiple domains including CUB, EGF, Keltch *etc;* and 80–90% homology to other insect/mosquito homologs. *Ac-atrn* gene was dominantly expressed in the young larvae and its expression was elevated in response to the fresh food supply in the starved larvae. Cold stress temporarily arrested the expression of *Ac-atrn* gene. In case of adult mosquitoes, olfactory and brain tissue showed relatively higher expression of *Ac-atrn* than reproductive organs. Although, starvation did not yield significant changes in olfactory tissues, but an ageing and nutritional stress modulated *Ac-atrn* expression in the brain tissue. Furthermore, a circadian rhythm dependent change in the expression of *Ac-atrn* of virgin and mated mosquitoes (both sexes), indicates that *Ac-atrn* might also have a pheromone guided role during swarm formation and mating behaviour.

Interpretation & conclusion: The relative expression profiling of *Ac-atrn* gene in the larvae during nutritional and cold stress suggested its possible role in mediating chemical communication towards the food source and in thermal regulation of young larvae. Similarly, it might have crucial regulatory role in the stress management and survival of adult mosquitoes. The results revealed that *Ac-atrn* gene is a global regulator of many physiological processes in mosquitoes including stress response and mating behavior and thus might be a potential target to design novel intervention strategy against mosquitoes.

Key words: *Anopheles*; *attractin;* mosquito; malaria; mating; stress; olfactory

INTRODUCTION

Attractin (*Atrn*) is a type I glycosylated protein, having multiple domains. It is widely expressed in different types of tissues in the body and facilitate cell adhesion[1-2]. In addition, it acts as a guidance molecule in regulating multiple physiological and pathological processes[1]. *Attractin* was first identified as an autosomal recessive gene, which suppresses *A*γ-induced pigmentation in mice[3]. It primarily exists in two forms, a transmembrane and a secreted form[4]. Both the secreted and membrane type *atrn* protein contains two epidermal growth factor (EGF) domains, a CUB domain, a C-type lectin domain, and two laminin-like epidermal growth factor domains. It is predicted that these functional domains are present in the extracellular portion of the membrane type *ATRN*[5]. It has been reported that the secreted form of *atrn* is more predominant in the body, that exists as serum glycoprotein. Earlier reports have suggested that *atrn* is also involved in crucial immune physiological process. It is synthesized by activated T-cells and released into the extracellular region, where it modulates the interaction between the monocytes and the T-cells and thus facilitates antigen presentation process effectively[1]. Membrane type *Atrn* has a novel function in myelination and has been found to prevent neuronal damage during oxidative stress[2]. Mutation of this gene is responsible for the zitter mutant phenotypes in mouse which includes the symptoms of hypomyelination and vacuolation in the central nervous system (CNS)[6]. It is also involved in hair pigmentation[7–8]. Both the membrane and the secreted form of *atrn* are found/present in humanshowever, only the membrane type has been identified in mice, where it is also reported to regulate the proper functioning of sperm in the testes[5]. Age-dependent progressive loss in functioning of *atrn* are responsible for testis vacuolation and diminished sperm function[5]. Further, a knockdown study of *atrn* gene in mice showed that it might be responsible for regulating other associated proteins in the sertoli cells of testes, which work cumulatively for the proper functioning of sperms[9].

In invertebrates, it was first identified as water-borne pheromone in the mollusc *Aplysia californica,* which is synthesized and secreted by the female reproductive organ to guide and attract sperms and facilitate sperm-egg interaction for successful fertilization within a distance of 10 m[10]. In *Aplysia*, *atrn* is a 58–60 kDa secreted protein which lacks the transmembrane domain and belongs to a member of multiple pheromone proteins, facilitating chemical communication, mate attraction, egg-sperm fertilization and many other functions[11].

Mosquitoes that transmit many infectious diseases are central to the control and management of vector borne diseases, causing heavy human health toll annually. Interfering their behavioural activities such as feeding, mating, breeding with novel approaches is valuable to counter attack the disease transmission. Pheromones, that are small peptides secreted by many tissues play a crucial role in mosquito's social behaviour and consequently their life cycle maintenance[12–14]. So far, there is very limited knowledge on the mosquito pheromones characterization, in principal due to challenges in *in vitro* identification, purification and characterization. In the present study, a unique transcript encoding the *atrn* homolog was characterized from the olfactory tissue of *Anopheles culicifacies* mosquito, the main rural malarial vector in India[15]. A comprehensive *in silico* analysis and extensive transcriptional profiling in response to feeding, mating and other stress responses provided evidences that *Ac-attractin* might have an important role in the management of neuro-olfactory regulation and stress management, enabling mosquito's successful survival.

MATERIAL & METHODS

Mosquito rearing & maintenance

The cyclic colony of *An. culicifacies*, sibling species A was reared and maintained under standard laboratory conditions at 28±2 °C and 80% relative humidity in central insectary facility as mentioned in previous studies[16-17].

Ethical statement

All protocols for rearing and maintenance of the mosquito culture were approved by ethical

committee of the National Institute of Malaria Research, New Delhi (NIMR Registration number: 33/1999/CPCSEA).

Tissue collection & RNA extraction

From the five min. cold (4 °C) anesthesized, 5–6-day old sugar fed adult male and female *An. culicifacies* mosquito's various tissues were collected according to the assays described below in the material and methods and in results. The tissues included olfactory organs (antennae, maxillary palp, proboscis and labium), brain, and reproductive organs (male reproductive organs: testes and male accessory gland; female reproductive organs: spermathecae and atrium). The tissues were dissected and collected in TRIzol. The developmental stages of *An. culicifacies viz.* egg, larvae (stages I – IV) and pupae were also collected in TRIzol reagent after removal of extra water through filter paper. Total RNA was isolated by the standard TRIzol method as described previously[16, 18].

Bioinformatic analysis

The putative *Ac-atrn* gene was identified as a partial contig, during analysis of olfactory tissue transcriptome data from the naive sugar fed adult female mosquitoes[19]. Initial BLASTX analysis against the NCBI NR database showed significant hits to the putative *atrn* like proteins of multiple mosquitoes, insects and other invertebrate species. To retrieve full-length *Ac-atrn* transcript, BLASTN analysis was performed against the genome predicted transcript database of the *An. culicifacies* mosquito, which is available at *www.vectorbase.org*. Domain prediction, multiple sequence alignment, and phylogenetic analysis were done using multiple softwares as described earlier[18].

External stress response of first instar larvae

To understand the response of *Ac-atrn* under the external stressed condition, the first instar larvae were kept overnight at 4 °C. For heat treatment, the larvae were exposed to 42 °C for 4 h. Then, to track the *Ac-atrn* expression under the nutritional stressed condition, the freshly hatched I instar larvae were taken and divided into three groups, each containing 30–40 larvae. The first group of larvae were collected immediately after hatching as control batch, the second group of larvae were kept overnight in starvation, without any food supply; and the third group was provided with 20–30 mg of a mixture of fish food and dog biscuit. After 24h, 30 starved larvae were collected in TRIzol and the remaining starved larvae were provided with food to recover from the nutritional stress. Following 24 h of maintenance, these remaining larvae were collected in TRIzol for relative gene expression analysis.

External stress response of adult mosquitoes

To expose the adult male and female mosquitoes with extrinsic stress, the adult mosquitoes were kept starved for overnight. Next day, the olfactory and brain tissue were dissected from starved and their respective sugar-fed mosquitoes (control). Further, a detailed time course experiment of the starvation assay was performed, and the brain tissue was dissected and collected in TRIzol to analyze the *Ac-atrn* expression in brain tissue during starvation.

Behavioural and molecular assay

To track the possible role of *Ac-atrn* in mating behaviour, an assay was designed monitoring the sex-specific changes of the behavioural activities occurring in response to day/night cycle in the 5–6 days old mosquitoes. As per the assay design, olfactory and reproductive tissues were collected from either virgin and/or mixed cage mosquitoes of both the sexes. For the assurance of mating success, an equal number of male and female virgin mosquitoes were mixed in a single cage at early morning (1000 h), and the tissues (both olfactory and reproductive) were collected at evening (1700 h) and next morning (1000 h) from the cage.

cDNA preparation & gene expression analysis

A 1 µg of total RNA was used to synthesize the first strand cDNA using Verso cDNA synthesis kit (Thermo Scientific, USA) as described in the manufacturer's protocol. Routine differential gene expression analysis

was carried out by RT-PCR. Relative gene expression analysis was done using SYBR green qPCR (Thermo Scientific, USA) master mix and Illumina Eco Real-Time PCR machine. The four step PCR cycle included an initial denaturation at 95 °C for 5 min, 40 cycles of 10 sec at 95 °C, 15 sec at 52 °C, and 22 sec at 72 °C. Fluorescence reading was recorded at 72 °C after each cycle. In final steps, PCR at 95 °C for 15 sec followed by 55 °C for 15 sec and again 95 °C for 15 sec were completed before generating melting curve. Reproducibility of the result was ensured by repeating the experiments with three independent biological replicates. Throughout the experiment, *RpS7* and *actin* genes were used as an internal control (as they are constitutively expressed in all conditions) and the relative quantification data were analyzed by $2^{-\Delta\Delta Ct}$ method[18]. Statistical analysis of differential gene expression was done using Student's *t*-test.

RESULTS

Identification, annotation and molecular characterization of Ac-atrn

A 1070bp long unique transcript (Accession# MF599469) has been identified during annotation of large-scale RNA-Seq transcriptomic database originating from the olfactory tissues of the adult female *An. culicifacies* mosquitoes[19]. A detailed *in-silico* analysis predicted a 3942 bp long full-length *Ac-atrn* transcript (ACUA004165-RA), encoding 1313 amino acid long peptide. A function prediction analysis unravelled that putative *Ac-atrn* is a multi-domain protein containing at least five domains. These include two calcium binding EGF domain, one cysteine rich Plexin repeat-PSI domain, one nitrile specific-PLN01293 and one extracellular CUB domain (Fig. 1a/ Table 1). A conserved domain architecture retrieval (CDART) tool search analysis predicted the presence of a similar but varying number of domain containing protein homologs in diverse organisms (Table-2), supporting their evolutionary conserved role.

In silico analysis of the full-length transcript, revealed that *Ac-atrn* is a single copy gene having six exons and five introns with 50bp flanking sequences at the 5′ upstream region and 50bp overhang at the 3′ downstream region (Fig. 1b). Multiple sequence alignment analysis of the *Ac-atrn* gene showed a high degree of conservation in the predicted domains namely, EGF, a cysteine rich repeat and CUB domain (Fig. 1c). Furthermore, a comprehensive phylogenetic analysis of *Ac-atrn* gene revealed a conserved relationship among blood feeding as well as non-blood feeding insects, and animals.

Food source stimulates attractin response in early larval development

To unravel whether *Ac-atrn* has any role during the aquatic development of the mosquito, a relative gene expression analysis was performed at different developmental stages of mosquito. A real-time PCR analysis showed relatively higher expression of *Ac-atrn* in the young L1 larvae when compared to egg and other developmental stages (Fig. 2a). Starvation of 10 h did not alter *Ac-atrn* expression, when compared to freshly emerged un-starved larvae of the same age. However, surprisingly, a two-fold (p <0.01) up-regulation of *Ac-atrn* was observed in the naïve as well as the starved larvae, when given fresh food supply prior and after starvation, respectively (Fig. 2b).

Cold stress arrests attractin expression

In order, to test whether external/environmental stress influence the *Ac-atrn* expression, the young larvae were exposed to an overnight cold stress, and their expressions were compared with unstressed larvae. Though, cold stress did not affect the survival of the larvae, depletion of *Ac-atrn* to a negligible level ($p < 0.002$) was noticed (Fig. 3). It was also observed that cold treatment temporarily arrested the motility of the larvae, which was recovered to the normal active stage when kept back at room temperature for 3–4 h. Along with the recovery of larval movement, *Ac-atrn* expression also reached to normal level after 3–4 h of the recovery phase. However, 4–5 h of heat exposure to the larvae at 42 °C did not alter *Ac-atrn* expression significantly ($p < 0.1$).

Nutritional stress may influence attractin response in the adult mosquito brain

A tissue specific relative expression analysis indicated that *Ac-atrn* constitutively expresses in the olfactory tissues, central nervous system and the reproductive organs of both male and female mosquitoes (Fig. 4a). But, ~2.5-fold higher level of expression was observed in the neuro-olfactory system than the reproductive tissues for both the sexes of naïve adult mosquitoes (Fig. 4a). An initial gene expression analysis of sugar fed versus blood fed olfactory system did not show any significant alteration in the *Ac-atrn* expression (Fig. 4b). Furthermore, *Ac-atrn* expression level in the sugar fed and starved (24 h) mosquito's olfactory system remains un-altered (Fig. 4c).

But surprisingly, a time dependent starvation significantly modulated *Ac-atrn* expression in the brain tissue of adult mosquitoes of both sexes (Fig. 4d, e). The male mosquitoes brain showed an early transcriptional response (6 h after starvation) of *Ac-atrn* gene ($p < 0.0001$) under nutritional stressed condition (Fig. 4d), whereas, female mosquitoes brain showed a delayed elevation of *Ac-atrn* at 30 h of starvation ($p < 0.0001$) (Fig. 4e) and the mortality rate of male mosquitoes was much higher than their female counterpart (Fig. 4f).

Age and sex specific olfactory response of attractin may influence mating behaviour

Given the multi-functional properties of *atrn*, it was further tested whether age dependent maturation affects the *Ac-atrn* response in mating behaviour of the mosquitoes. To examine this relationship, an age and sex specific relative expression analysis of *Ac-atrn* gene was performed in the olfactory and brain tissue of *An. culicifacies* mosquito. A significant and continuous increase (~6 fold for female OLF and ~3.5 fold for male OLF) in *Ac-atrn* expression was observed till the 7th day in the olfactory tissue of both virgin male and female mosquitoes (Fig. 5a, b). However, as compared to the olfactory tissue brain did not show any significant modulation, except an initial increase of *Ac-atrn* level in the aging adult female mosquitoes (Fig. 5c)

A circadian dependent transcriptional profiling indicated that mating status did not alter the *Ac-atrn* expression in the reproductive tissue of both the sexes (Fig. 5d). However, a significant (>2.5-fold) change in the *Ac-atrn* expression in the mated mosquito's olfactory system provides an evidence that *attractin* may facilitate pheromone guided male-female courtship behavior (Fig. 5e).

DISCUSSION

The functional characterization of novel genes regulating different physiological responses in mosquitoes, is very important for targeted control of mosquito population. To unravel the smell detection mechanism of *An. culicifcaies* mosquito a comprehensive RNA-Seq analysis has been performed19. Identification of a unique gene named *attractin,* from the naïve sugar fed mosquitoes olfactory tissue transcriptome data, prompted us to investigate its role in the mosquito *Anopheles culicifacies*. Existing literature search demonstrated that *atrn* gene plays crucial role in immunity, neuro-physiology and reproduction in vertebrates like human and mice[1–2, 5–6]. *Aplysia californica* is the only invertebrate species where *atrn* gene guides sperm motility towards distantly located eggs for successful fertilization[10–11]. However, its role has not been investigated in any of the mosquito species. To resolve its possible evolutionary functions a detailed *in-silico* analysis was performed. A comprehensive phylogenetic analysis showed a conserved relationship among all these organisms predicting that *atrn* gene may have similar multi-physiological role in the mosquito *An. culicifacies*.

Initial developmental expression profiling of *Ac-atrn* gene indicated that an increase in *atrn* expression in emerging young larva may be important to taste, smell and move towards food sources. Although, nutritional stress do not influence the expression of *Ac-atrn* in the larvae, however, a food supply may accelerate its expression, possibly to regulate the larval movement towards the food source. This may be one of the unique features of *atrn,* having the water borne chemical communication (pheromone mediated) property, similar to

sperm egg interaction in *Aplysia calfornica*[10]. Furthermore, the exact role of *Ac-atrn* in the regulation of thermal stress is still not clear, however our study showed that cold stress may temporarily arrest *atrn* expression possibly to minimize the energy loss and hence facilitate its survival.

A ~2.5-fold higher level of expression was observed in the neuro-olfactory system than the reproductive tissues for both the sexes of naïve adult mosquitoes, suggesting its possible role in mosquito's behavioural biology and stress management. Though, a different food source and nutrient deprivation have only nominal effect in the *Ac-atrn* expression, but, starvation causes significant modulation of *Ac-atrn* expression in the brain tissue of adult mosquitoes of both sexes. Furthermore, the time-course dependent relative expression data indicated that male brains are more susceptible to starvation induced neuronal damage as compared to female brains and thus consequently affect the mortality rate of male mosquitoes. Taken together, it is hypothesized that an early up-regulation of *Ac-atrn* in the male brain may be an attempt to protect the brain cells from fasting induced oxidative damage and consequently neuronal degeneration and death[21]. It is well known that human and other vertebrate's brain is a highly metabolic organ in the body which consumes a large amount of energy in the form of nutrition/food[20]. Although, brain is highly susceptible to oxidative damage due to the abundance of oxidizable material in the plasma membranes of neural cells, however, food deprivation has an added value which causes a failure in the oxidative stress management and thus leads to brain cells degeneration and death[21]. Further a >10-fold elevation of *Ac-atrn* in the female brain during later stage of starvation suggested that female mosquitoes can survive for a longer period of time without any food source and thus are more adaptive to adverse environmental conditions which favour their evolution and existence. A continuous elevation of *Ac-atrn* gene was observed in the olfactory system, whereas brain showed poor modulation, except an initial increase, of *atrn* gene expression in aging mosquitoes. These data indicate that olfactory *Ac-atrn* may have an important role in the regulation of mosquito behavioural events and may regulate mating behaviour during early adulteration age. Thus to test the possible role of *Ac-atrn* in mating behaviour a circadian dependent transcriptional profiling was performed. The data evidenced that *Ac-atrn* may facilitate pheromone guided male-female courtship behaviour. For a successful mating, mosquitoes need to deal the complex events of swarm formation and courtship engagement, ending with successful insemination; a process possibly guided by natural dysregulation of *quick-to-court* protein in the olfactory system of the mosquitoes[22]. Though it is yet to be clarified that how active swarm formation and courtship engagement is guided, but the present data suggested that *Ac-atrn* may have a key role to attract the couples during swarm formation, which is actively commenced on the onset of the sunset (1700 h).

CONCLUSION

Among millions of insects, mosquitoes are evolved with extra specialization of sensory tissues that facilitate them to feed, mate, breed and adapt in diverse ecologies. In vertebrates and few invertebrates, a multi-domain proteins *attractin*, facilitate many physiological functions and thus have been regarded as a potential therapeutic target for many neuro-regulatory and sexual disorders. Under multiple innate physiological status of mosquito, the transcriptional response of *attractin* homolog *Ac-atrn* gene was evaluated that was originally identified from the olfactory system of *An. culicifacies*. The comprehensive *in silico* analysis and transcriptional regulation indicates that *Ac-atrn* not only supports neuro-olfactory associated physiological functions but may also play a crucial role in courtship engagement behavioural responses (Fig. 6).

Conflict of interest: The authors declare no conflict of interest.

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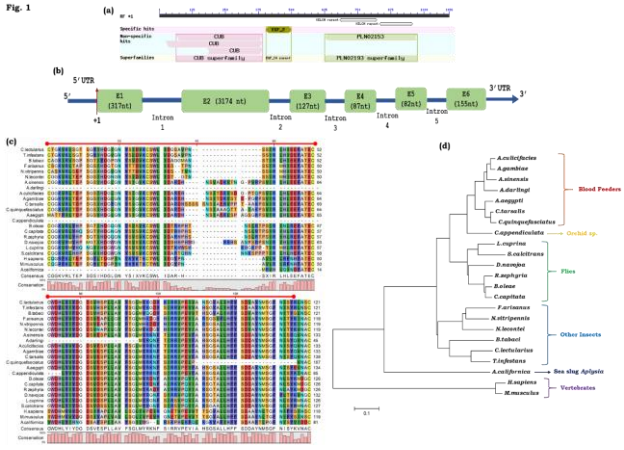

Fig. 1: Molecular analysis of *An. culicifacies attractin (Ac-atrn)* gene: (a) Domain architechture of *Ac-atrn* gene. (b) Schematic representation of the genomic architecture of the *Ac-atrn gene.* Five green colored boxes indicates the exons, blue line denotes the introns and +1 mark the transcription initiation site. The size of the exons and introns correspond to the size of the boxes and lines. (c) Multiple sequence alignment of a segment of *An. culicifacies attractin* with other mosquitoes, flies, invertebrates and vertebrates homologs showed high degree of conservation in the amino acid sequence. One of the CUB domain is highlighted with red line. (d) Phylogenetic relationship of *Ac-attractin* indicates that *An. culicifacies attractin* is clustered within the mosquito domain and have much greater similarity with mosquito *attractin* than flies and other insects. Human, *Mus musculus* and invertebrate *Aplysia californica attractin* sequence were also considered in this analysis for out-group clustering,

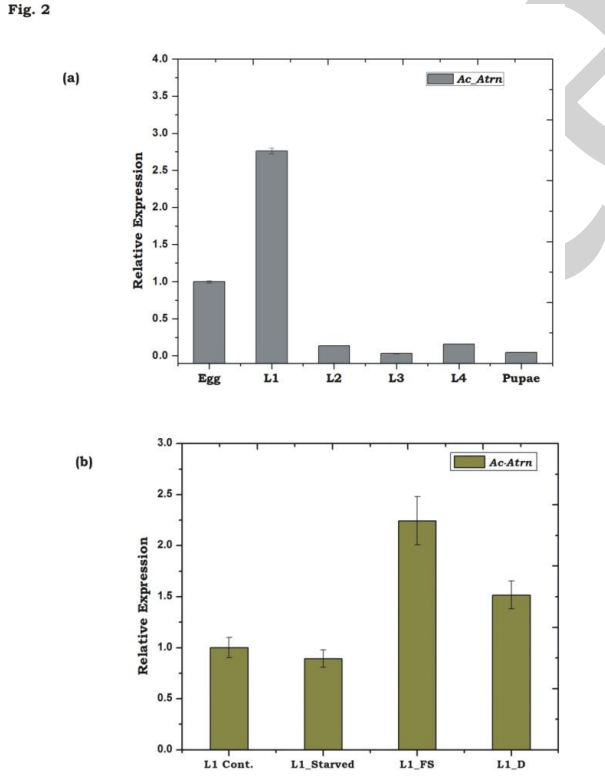

Fig. 2: Transcriptional profiling of *attractin* gene in *An. culicifacies* developmental stages. (a) Real-Time PCR mediated developmental expression of *Ac-atrn* in *A. culicifacies.* L1-L4: Larvae stage 1 – stage 4. (b) Relative expression analysis of *Ac-atrn* under food stressed conditions in the first instar larvae.

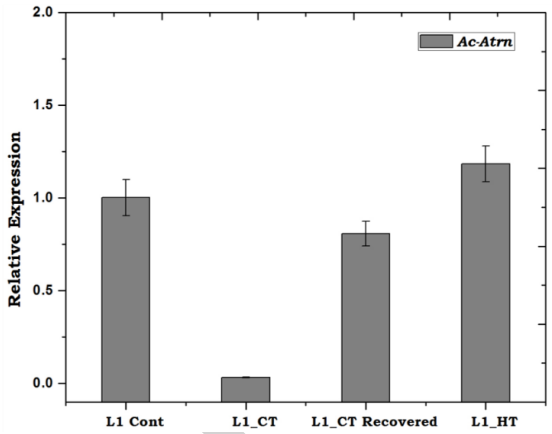

Fig. 3: Differential gene expression analysis of *Ac-atrn* gene in the first instar larvae under temperature stressed conditions.

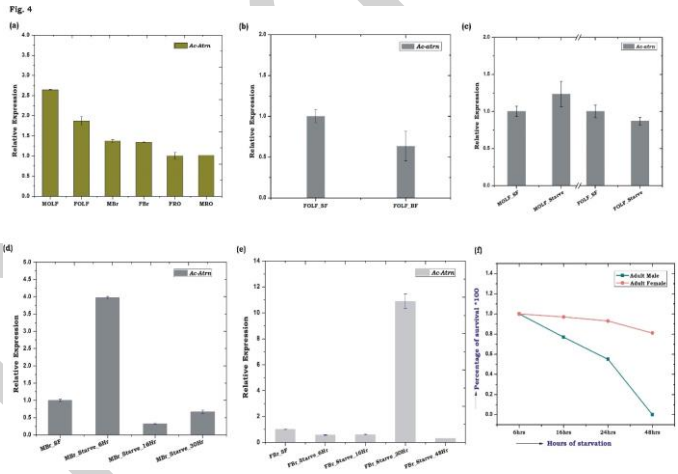

Fig. 4: Tissue specific transcriptional behaviour of *Ac-atrn*. (a) Tissue specific relative expression analysis of *Ac-atrn*. MOLF: Male olfactory tissue (Antennae, maxillary palp and proboscis); FOLF: female olfactory tissue; MBr: Male brain; FBr: female brain; MRO: Male reproductive organ; FRO: Female reproductive organ. (b) *Ac-atrn* expression pattern in sugar fed and blood fed olfactory tissues. FOLF_SF: sugar fed female olfactory tissue; FOLF_BF: Blood fed female olfactory tissue. (c) Transcriptional response of *Ac-atrn* in the olfactory tissues of both male and female mosquitoes under food deprived condition. MOLF_SF: sugar fed male olfactory tissue; MOLF_starve: 24hrs. starved male olfactory tissues (Same for the females). (d) A time dependent transcriptional profiling of *Ac-atrn* in the brain tissues of male *An. culicifacies* mosquitoes under food deprived condition. MBr_SF: Male brain dissected from sugar fed mosquitoes; MBr_Starve_6Hr: Male brain dissected after 6hrs of starvation (Same in case of other time points). (e) A time dependent transcriptional profiling of *Ac-atrn* in the brain tissues of female mosquitoes under food deprived condition. FBr_SF: female brain dissected from sugar fed mosquitoes; FBr_Starve_6Hr: Female brain dissected after 6hrs of starvation (Same in case of other time points). (f) Survival curve of 3-4 days old adult male and female mosquitoes under food deprived conditions.

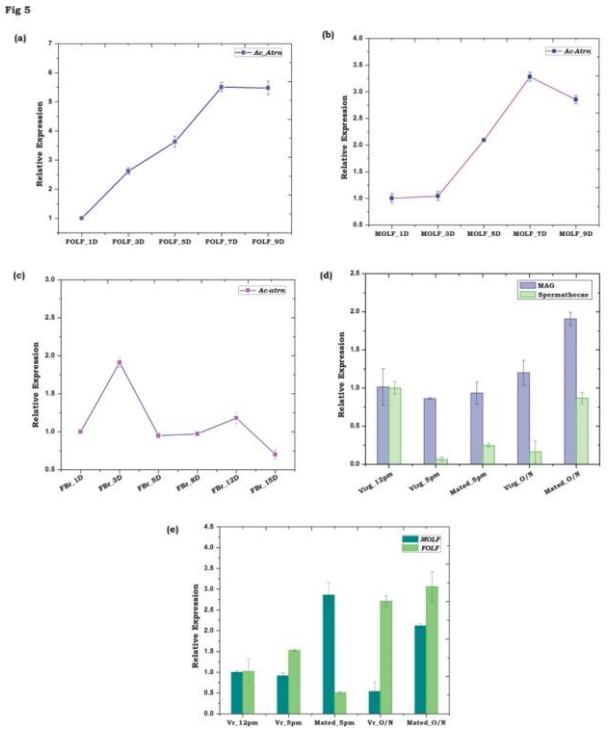

Fig. 5: Age and circadian clock dependent transcriptional response of *Ac-atrn* transcript in male and female *A.culicifacies* mosquitoes. (a) Age dependent relative transcriptional regulation of *Ac-atrn* in female mosquito olfactory system. FOLF-1D: Female mosquito of 1Day old (Similar pattern for others). (b) Transcriptional response of *Ac-atrn* in male mosquito according to their age. MOLF-1D: Male mosquito of 1 Day old (Similar pattern for others). (c) Age dependent relative transcriptional profiling of *Ac-atrn* in female mosquitoes' brain. FBr-1D: Female mosquitoes' brain of 1Day old (Similar pattern for others). (d) Circadian time dependent and the mating status dependent expression pattern of *Ac-atrn* in the reproductive organ of both male and female mosquitoes. Virg_12pm: Virgin mosquitoes dissected at 12 pm; Mated_5pm: Mated mosquito dissected at 1700 hr.; MAG: Male accessory gland; Virg_O/N and Mated_O/N: Virgin and mated mosquito dissected after overnight exposure to each other respectively. (e) Circadian time dependent and the mating status dependent expression pattern of *Ac-atrn* in the olfactory tissue of both male and female mosquitoes.

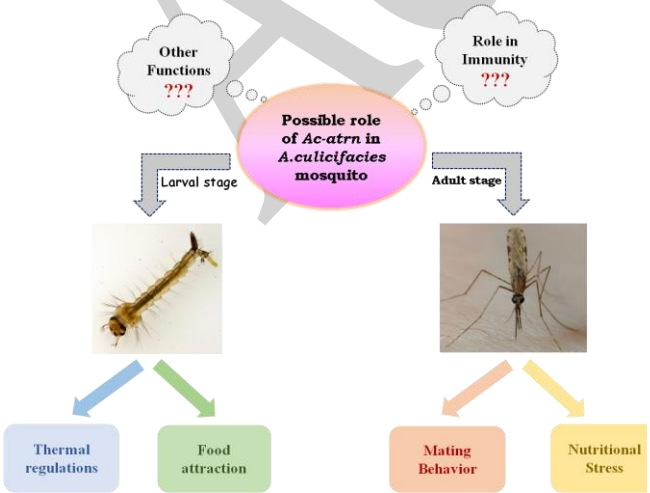

Fig. 6: Proposed hypothesis for the possible functions of *attractin* gene in the mosquito *An. culicifacies*.

Table 1. Domain architecture of *Ac-atrn* gene.

S. No.	Domain Description	Start site	End site
1.	CUB domain	97	233
2.	CUB domain	98	218
3.	CUB domain	101	228
4.	CUB domain	101	230
5.	CUB domain	101	231
6.	EGF-like domain	67	99
7.	EGF-like domain	70	99
8.	EGF-like domain	232	265
9.	EGF-like domain	265	301
10.	EGF-like, conserved site	87	98
11.	EGF-like, conserved site	289	300
12.	Kelch-type beta propeller	302	508
13.	Kelch-type beta propeller	529	945
14.	Laminin EGF domain	942	986
15.	Laminin EGF domain	989	1035
16.	PSI domain	655	706
17.	PSI domain	723	778
18.	PSI domain	784	825
19.	PSI domain	827	877
20.	PSI domain	885	940
21.	Plexin repeat	828	876

Table 2. Identity match of *Ac-atrn* with other mosquitoes, insects and vertebrate homologs:

Species name	Identity	Query	E-value
Anopheles	94%	99%	0.0
Aedes albopictus	84%	94%	0.0
Aedes aegypti	83%	94%	0.0
Culex	80%	95%	0.0
Anopheles	89%	85%	0.0
Bactrocera	63%	94%	0.0
Ceratitis	63%	94%	0.0
Drosophila	62%	94%	0.0
Musca	61%	94%	0.0
Homo sapiens	36%	71%	3e-136
Mus musculus	36%	70%	2e-136
Aplysia	38%	88%	0.0

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Biogenic Amines in Shaping Mosquito Behavior: A Biomolecule having Pharmacological Significance

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ABSTRACT

Despite of the lots of control measures against mosquitoes, the prevalence of malaria, dengue fever and other mosquito borne diseases have reached at an alarming level. Continuous climate alteration and generation of insecticide resistance are the primary reason for the vector emergence. Thus, developing new molecular tools and unraveling unique molecular targets for designing of novel mosquito repellent are urgently needed. Here, in this review we have highlighted the functions of two unique biogenic amines of mosquitoes and other insects. Further, functional and structural characterization of these two amines, viz. octopamine and tyramine, and their respective receptor might accelerate the scheming of novel chemical molecule to minimize the mosquito population growth.

Keywords: Malaria; Population growth; Elimination program

INTRODUCTION

Mosquitoes are one of the potent carriers of many debilitating vector borne diseases and cause millions of death each year. Changing in the environmental conditions and increased insecticide resistance are posing a challenge to control the mosquitoes [1]. Despite of growing awareness of malaria elimination program, launched by WHO, still there are significant increase in the cases vector borne diseases such as malaria, dengue in recent years in India [2]. In this genomic era, a wide scientific community is engaged to unravel molecular basis of different aspects of mosquito biology, essentially needed if new molecular tools are to be developed rapidly. One of the key strategies may rely to disrupt the adult female mosquito-human exposure. Thus unlocking and identifying crucial/novel molecular factors regulating the complex host seeking and blood feeding behavior could be an important step to establish such innovative tools [3,4].

Behavior of any organism from lower to higher organism is not a simple and single event, but it is managed by a complex but beautifully orchestrated series of events [5]. Though higher animals took advantage of having multiple sensing organs *viz*. visual, smell, auditory etc., but the total insect community is solely dependent on their smelling power to initiate each of their behavior [6-8]. Behavioral initiation through detection of chemical odors is further shaped by the finely tuned nervous system [9]. Different neurotransmitters, their cognate receptors includes diverse a diverse nature of neuromodulators such a neuropeptides and biogenic amines which synergistically coordinate to configure the insect's behavior [10]. In case of blood feeding insects, the evolutionary force also gives them the specialty of having blood meal from vertebrate host, a unique behavioral response, tightly regulated by both internal physiological cum nutritional needs as well as by external stimuli [11-14]. In comparison with other blood feeding insects, mosquitoes take much attention for its disease transmission ability due to its opportunistic and unique nature of blood feeding ability which make them the most dangerous disease transmitting vector [15,16].

Like other insects, each behavioral response of mosquitoes *viz*. hosts seeking, feeding, mating, oviposition is tightly regulated by olfaction followed by neuronal decision making genetic abilities [11,17]. However, the rate of success heavily dependent on detecting information from the surroundings, analyzing and processing them for rapid

execution of targeted behavior [7]. The plasticity in shaping of each of these behaviors is locked in the type of neuro-signaling mechanism, well-coordinated by associated learning process [18]. Though, the role of classical neurotransmitters in relaying information in the nervous system relatively has well been studied in other insects but the crucial role of other neuro-modulators such as neuropeptides and small biogenic amines are yet to unraveled. Thus in this review we aimed to give some glimpse of current knowledge over biogenic amines and their role in neuro-mediated mosquitoes behavioral regulations.

SYNTHESIS OF BIOGENIC AMINES

Biogenic amines are evolutionary conserved molecule which not only function in neuro-signaling but also play crucial role in several physiological functions [19]. Most of amines are common in both vertebrates and invertebrates (Dopamine, serotonin and histamine), but some biogenic amines are synthesized preferentially in either vertebrate (epinephrine and norepinephrine) or in invertebrates (tyramine and octopamine) to perform some specialized physiological functions [20,21]. Three different amino acids are the source of synthesis of different biogenic amines through multiple enzymatic reactions [20]. Tyrosine acts as a raw material for the synthesis of dopamine in both vertebrates and invertebrates. In addition to that, invertebrates have specialized biochemical pathway for the synthesis of tyramine and octopamine from tyrosine itself [20]. Next, serotonin is synthesized from tryptophan and histidine is the source amino acid for histamine in both vertebrates and invertebrates [20]. Thus, targeting the insect's specific pathway for the synthesis of tyramine and octopamine and blocking the tyraminergic and octopaminergic receptors mediated signal transduction cascade by some chemical means may be one of the alternative strategy to manipulate vector population which also has the benefit of exemption of off-target effects [20,22].

Biological Functions of Biogenic Amines

Valuing the central role of biogenic amines towards managing complex behavioral responses, here we summarize and update the knowledge of the insect/mosquito specific biogenic amines functions.

Octopamine:

Octopamine is broadly distributed in the insect's nervous system where it is called "flight and fight hormone" due to its ability of stress management [23]. Previous literature indicated that octopamine may play crucial role in non-specific arousal system of insects, where it can judge the stressful situation and facilitate in decision making of either fight or to escape [24]. In the central nervous system, it involved in the desensitization of olfactory stimuli, facilitate learning and memory and thus affect the adaptation [25,26]. By modulating the behavioral responses to attractant (pheromone), octopamine alters the mood of the animal [20]. Apart from the neuro-olfactory regulations, it is also found to present in the non-neuronal tissues such as hemolymph, muscle fiber, sense organs etc. [20,27]. Octopamine manages energy metabolism and homeostasis when present in the hemolymph, by regulating the breakdown of sugars and lipids [27]. In the peripheral organ of neuromascular junctions it affects intramascular protein synthesis which is concomitantly regulate muscular rhythm [20,28]. Surprisingly, an availability of considerable number of evidences predicting octopamine functions in other insects, but its role is poorly analyzed in mosquitoes. Thus, unraveling the molecular functions and mechanism of octopamine in the behavioral regulations in mosquitoes might open new opportunities to develop novel control strategies for mosquitoes with minimal adverse effect on the environment [29].

Tyramine:

Though, tyramine is the precursor molecule of octopamine but it has a significant role in insect body and it is believed that both octopamine and tyramine functions antagonistically, actions similar to adrenalin and noradrenalin in vertebrates [20]. For example, behavioral response to attractive odor is mediated by octopamine whereas behavioral response to general odorants is facilitated by tyramine [20]. Previous study by Kutsukake et al. suggested that tyramine receptor mutation impaired the olfactory responses of fruit fly *Drosophila melanogaster*, and these mutant flies are unable to avid repellent odors [30]. Except that, both octopamine and its precursor tyramine are known to involve in the regulation of larval locomotory behavior aversively [20]. Increase in the tyramine level reduces the speed of larval crawling movement [31,32]. Tyramine also modulates the activity of peripheral muscle of the reproductive system and legs of insects [33]. In case of mosquitoes, the octopaminergic and tyraminergic signaling was found to be crucial for oviposition and egg melanization but the other behavioral and physiological regulations of tyramine is remains to unravel [22].

Dopamine:
Dopamine is an important multifunctional neuromodulator, neurotransmitter and neuro-hormone both in invertebrates and vertebrates. In invertebrates, it is reported to have functions in olfactory reception and was found to influence the age dependent retardation of olfactory sensitivity [20,34]. Dopamine also play crucial role under nutritional stressed conditions, where the sensitivity of dopamine receptor increases against sucrose under starvation [20,35]. Studies also suggest that the regulation of amount of sleep and wakefulness is tightly modulated by dopamine and associated. A recent study by Kanta Terao et al. suggested that the neurons that are sensitive to dopamine play pivotal role in aversive learning in crickets [36]. It also has role in locomotion, courtship and development [20]. In mosquitoes, dopamine is directly related to host seeking behavior where it was found that increase in dopamine level reduces host finding activity [37].

Serotonin:
Serotonin is other most abundant monoamines in the gastrointestinal tract but also distributed throughout the animal body where it plays crucial multifunctional role [38]. In may insects, including mosquito's serotonin neurons are found to innervate in the midgut and the crop where it regulates the movement of the food through the gut [38]. Increase in the level of serotonin in the hemolymph of insects showed a reduction in the meal size [20,38]. In addition to that, serotonin functions in co-ordination with glutamate and was found to elevate the heart contraction rate in the mosquitoes [39]. Serotonin was also found to influence the feeding behavior of larvae of *Ades aegypti* mosquitoes [40]. Apart from that, previous study showed that serotonergic neurons that are innervated on the salivary gland of mosquitoes play crucial role in the regulation of salivation [41,42]. Recently, serotonin was also found to regulate hemocyte mediated immune response of the caterpillar *Pieris rapae* [43].

Histamine:
In comparison with other insect's biogenic amines the role of histamine has not been studied in detail. It acts as major neurotransmitters that are released in the photoreceptor cells after light exposure [20]. It has a crucial role in the communication between interneurons. Thus, histamine is considered as a crucial molecule however its role in sensory transduction in the visual perception and maintaining the interneuron connections is yet to be elucidated [20].

Prerequisite for Analyzing the Pharmacological Aspects of Biogenic Amine
Biogenic amines are small biomolecules which initiated their functions after binding with their respective receptors. The biogenic amine receptors predominantly belong to GPCR family. The signal transduction cascade that is activated after binding of the amines with their cognate receptors either is mediated by the synthesis of cAMP or by elevating the concentration of Ca^{2+} ion that are present on a specific location [20]. From the current literature, it is plausible to propose that biogenic amines and their receptors coordinate synergistically in the regulation of neuronal signaling, both in peripheral and central nervous system. Though, the roles of five different biogenic amines were extensively studied in other insects but unraveling their crucial function is very much needed in mosquitoes (Figure 1).

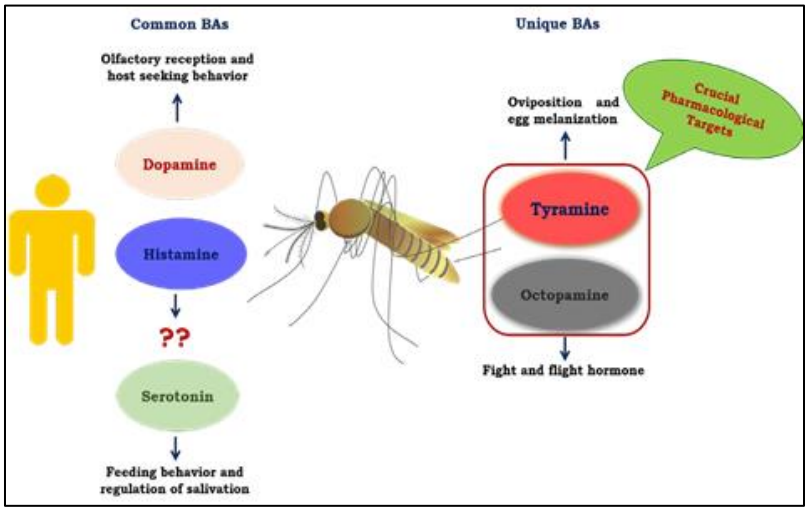

Figure 1: Schematic representation of the biogenic amines and their possible roles

Thus, the molecular characterization of biogenic amine receptors and their downstream molecules are prerequisite to design novel molecular tool for the management of diverse vectors including mosquitoes. Combination of experimental studies for function prediction analysis with the bioinformatics based structure prediction analysis coupled with the designing of novel chemical antagonists will not only improve our understanding of mosquito biology but also be an alternative strategy for mosquito control.

CONCLUSION

Dopamine, histamine and serotonin are the common biogenic amines in both vertebrates and invertebrates (mosquitoes), whereas tyramine and octopamine are insect's specific amines. Thus, octopamine and tyramine can be used as crucial molecular targets in future, for designing of novel chemical/pharmaceutical molecule to control mosquito population.

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Sex specific molecular responses of quick-to-court protein in Indian malarial vector *Anopheles culicifacies*: conflict of mating versus blood feeding behaviour

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Abstract

Understanding the molecular basis of mosquito behavioural complexity plays a central role in designing novel molecular tools to fight against their vector-borne diseases. Although the olfactory system plays an important role in guiding and managing many behavioural responses including feeding and mating, but the sex-specific regulation of olfactory responses remain poorly investigated. From our ongoing transcriptomic data annotation of olfactory tissue of blood fed adult female *An. culicifacies* mosquitoes; we have identified a 383 bp long unique transcript encoding a *Drosophila* homolog of the quick-to-court protein. Previously this was shown to regulate courtship behaviour in adult male *Drosophila*. A comprehensive *in silico* analysis of the *quick-to-court* (*qtc*) gene of *An. culicifacies* (*Ac-qtc*) predicts a 1536 bp single copy gene encoding 511 amino acid protein, having a high degree of conservation with other insect homologs. The age-dependent increased expression of

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putative *Ac-qtc* correlated with the maturation of the olfactory system, necessary to meet the sex-specific conflicting demand of mating (mate finding) versus host-seeking behavioural responses. Sixteen to eighteen hours of starvation did not alter *Ac-qtc* expression in both sexes, however, blood feeding significantly modulated its response in the adult female mosquitoes, confirming that it may not be involved in sugar feeding associated behavioural regulation. Finally, a dual behavioural and molecular assay indicated that natural dysregulation of *Ac-qtc* in the late evening might promote the mating events for successful insemination. We hypothesize that *Ac-qtc* may play a unique role to regulate the sex-specific conflicting demand of mosquito courtship behaviour versus blood feeding behaviour in the adult female mosquitoes. Further elucidation of this molecular mechanism may provide further information to evaluate *Ac-qtc* as a key molecular target for mosquito-borne disease management.

Keywords: Ecology, Evolution, Genetics, Zoology

1. Introduction

Mosquitoes are the vectors for many deadly infectious diseases including malaria, dengue, chikungunya, zika fever and yellow fever claiming a few hundred million lives annually. An adult female mosquito transmits the pathogens from an infected vertebrate host to a healthy host during a blood meal. The control of mosquito-borne diseases is still dependent on the use of chemical insecticides to suppress mosquito population or to interrupt mosquito-human interaction. However, the rapid emergence of insecticide resistance poses a challenge to control vector populations effectively. An alternative to overcome this challenge includes designing molecular tools to interfere the complex feeding and mating behavioural properties [1]. Compared to the vast research concentrated on female mosquitoes, male mosquito biology is rather less explored possibly due to its indirect influence on parasite transmission. Males induce several post-mating behavioural changes in females, which also includes the blood feeding behaviour. Thus, male mosquitoes maintain the unbroken chains of the mosquito life cycle and significantly contribute in transmitting diseases in an indirect way. In nature, both males and females feed on nectar sugar for their regular metabolic energy source. Only adult female mosquitoes take blood meal to fulfil the requirement of extra nutrients for their egg maturation. However the molecular basis of evolution and adaptation of dual feeding behaviour i.e. nectar sugar versus blood meal in adult female mosquitoes is not fully understood [2, 3]. Likewise, demystifying the process through which mosquitoes manage complex mating behavioural events i.e. swarm formation, suitable mate finding and successful aerial coupling is yet a major challenge to entomologists [4, 5, 6, 7]. A few molecular markers linked to mating behaviour have been characterized in *Drosophila melanogaster* [8, 9, 10]. But, the

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unavailability of any molecular marker regulating the complex mating behaviour in mosquitoes restricted our understanding.

Previous studies have indicated that neuro-olfactory system of mosquitoes regulates many complex behavioural responses such as mating, host seeking and blood feeding [11, 12, 13, 14]. Current evidence indicates that both male and female mosquito's olfactory system encodes a fairly similar number of proteins [15, 16], but how sex-specific olfactory proteins manage the conflicting demand of feeding and/or mating behaviour is not known. Modest changes occurring in the olfactory repertoire in response to blood feeding in adult female mosquitoes have been reported by various groups [17, 18]. Therefore, we hypothesize that regulation of some of the sex-specific unique genes may facilitate and manage similar functions e.g. mate partner location by adult male/female mosquitoes or host finding for blood feeding by adult female mosquitoes.

An. culicifacies is one of the major rural vectors in India accounting for more than 65% of malaria cases. Control of this mosquito species has become worse due to the rapid emergence of multiple insecticide resistance [19]. In an effort to understand the complex feeding behaviour of adult *An. culicifacies* female mosquito [3], we have identified a unique transcript from the olfactory system of the blood-fed mosquito, encoding the 'quick-to-court' (qtc) protein. *An. culicifacies* qtc protein is a homolog of *Drosophila* coiled-coil qtc (Q9VMU5) protein and shown to play an important role in driving the male courtship behaviour. In *D. melanogaster,* it is predominantly expresses in olfactory organs, central nervous system and male reproductive tract [20, 21]. Mutations in the *Dm-qtc* not only caused the males to show elevated levels of male-male courtship, but also favoured abnormally quick courtship when placed in the presence of a virgin female [18]. Recently, a qtc homolog has also been identified from whole body transcriptome of the insect *Bactrocera dorsalis* [22, 23] but yet its function is yet to be characterized.

A significant modulation of this unique transcript, *Ac-qtc* in the olfactory tissue (~5 fold up-regulation) in response to blood feeding prompted us to investigate its possible role in managing sex-specific conflicting demands of 'mate choice' and/or 'food choice' in the mosquito *An. culicifacies*. A comprehensive *in silico* function prediction analysis and extensive transcriptional profiling established a possible correlation that *Ac-qtc* might play a crucial role in the regulation and coordination of mosquito sex-specific behaviours.

2. Materials and methods

Fig. 1a represents a technical overview and workflow of the experiments to demonstrate the possible sex-specific role of *Ac-qtc* in adult *An. culicifacies*.

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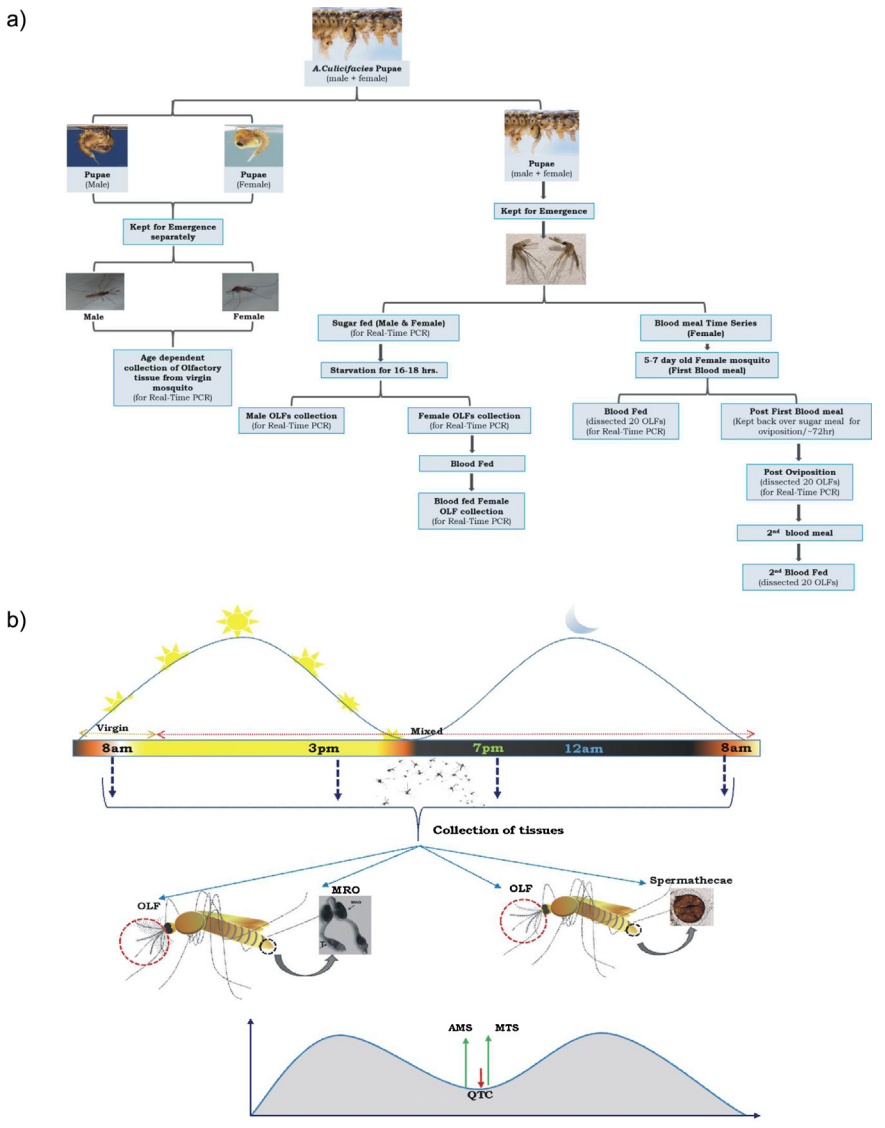

Fig. 1. Technical designing and experimental work flow: (a) Experimental overview to demonstrate the possible role of *Ac-qtc* in mosquito behavioural regulation; (b) Pictorial presentation of the assay designed to correlate the function of *Ac-qtc* in the mating success of *A.culicifacies* mosquito. OLF: Olfactory; MRO: Male reproductive organ.

2.1. Mosquito rearing and maintenance

The cyclic colony of *An. culicifacies*, sibling species A was reared and maintained in standard laboratory conditions at 28 ± 2 °C and 80% relative humidity in central insectary facility as mentioned previously [3, 24]. All protocols for rearing and maintenance of the mosquito culture were approved by ethical committee of the institute.

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2.2. Tissue collection and RNA extraction

From the ice anesthesized adult male and female *An. culicifacies* mosquitoes various tissues were collected. This included olfactory tissues (including antennae, maxillary palp, proboscis and labium), brain, reproductive tissues (male reproductive organ includes testes and male accessory gland; female reproductive organ consists of spermathecae and autrium) and legs. The tissues were dissected and collected in trizol. The developmental stages of *An. culicifacies* viz. egg, larvae (stages I – IV) and pupae were also collected in trizol after removal of extra water through filter paper. Total RNA was isolated by the standard trizol method as described previously [3, 25].

2.3. Bioinformatic analysis

The putative *Ac-qtc* was identified as a partial cDNA from olfactory tissue cDNA library sequence database of the blood fed adult female mosquito (unpublished). Multiple BLAST analysis against mosquito draft genome and other transcript database were done using open source analysis tools available at www.vectorbase.org. Domain prediction, multiple sequence alignment, and phylogenetic analysis were done using multiple softwares as described earlier [25].

2.4. Behavioural and molecular assay

To track the possible role of *Ac-qtc* in mating behaviour, an assay was designed (Fig. 1b) favouring sex-specific changes of the behavioural activities occurring in response to day/night cycle in the 5–6 days old mosquitoes. As per assay design, we collected olfactory and reproductive tissues from either virgin and/or mixed cage mosquitoes of both the sexes. For the assurance of mating success, we mixed an equal number of male and female virgin mosquitoes in a single cage at early morning (0800 h) and collected tissues at 1500 h, 1900 h, and overnight/0800 h next morning from the same cage. To test and validate the completion of insemination process, we profiled and compared the expression of two independent sperm-specific transcripts in the spermathecae of adult female mosquitoes. Previously characterized sperm-specific genes (AMS/FJ869235.1and MTS/FJ869236.1) of the mosquito *An. gambiae* [26] were queried to search and select sperm-specific homologs from the draft genome database of the mosquito *An. culicifacies*. The identified *Ac-ams* (ACUA010089) and *Ac-mts* (ACUA014389) transcripts sequences were used to design RT-PCR primers (See supplemental data for gene and primer sequence).

2.5. cDNA preparation and gene expression analysis

1 μg of total RNA was used to synthesize the first strand cDNA using Verso cDNA synthesis kit (Thermo Scientific) as described in the manufacturer protocol.

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Routine differential gene expression analysis was performed by the RT-PCR. Relative gene expression analysis was done using SYBR green qPCR (Thermo Scientific) master mix and Illumina Eco Real-Time PCR machine. The four step PCR cycle included 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 reading was recorded at 72 °C after each cycle. In final steps, PCR at 95 °C for 15 sec followed by 55 °C for 15 sec and again 95 °C for 15 sec were completed before driving a melting curve. Reproducibility of the result was ensured by repeating the experiments with three independent biological replicates. Throughout the experiment, *actin* gene was used as an internal control and the relative quantification data were analyzed by $2^{-\Delta\Delta Ct}$ method [27]. Statistical analysis of differential gene expression was done using Student's t-test.

3. Results and discussion

3.1. Identification, annotation and molecular characterization of *Ac-qtc*

To identify the differentially expressed genes in naïve sugar fed *vs.* blood fed olfactory system (OLF) of *An. culicifacies* mosquito, currently we are annotating large-scale RNA-Seq transcriptomic databases (unpublished). During this annotation, we identified a unique transcript from blood fed olfactory transcriptome encoding *Drosophila* homolog of quick-to-court (qtc) protein, which plays a crucial role in many aspects of male mating behaviour. BLASTX analysis of the partial 383 bp long transcript showed 59% identity with the *qtc* homolog of *Drosophila melanogaster*, but no putative conserved domain was identified. To retrieve full-length *An. culicifacies qtc* transcript, we performed BLASTN analysis against *An. culicifacies* genome and transcript databases using the partial transcript as a query sequence. Comparative alignment analysis of the partial and full-length transcript indicated that the identified 383 bp putative *Ac-qtc* transcript lacks both 5′ and 3′ sequences. The detail *in silico* analysis of 1536 bp long full-length *qtc* transcript (ACUA027268) encoding a 511 amino acid long protein showed coiled-coil domain signature at the 3′ end of the sequence. *Ac-qtc* is a single copy gene, comprised of a 50 bp 5′ UTR region followed by five exons and four introns followed by a 50 bp 3′UTR region as shown in Fig. 2a. A comprehensive primary structural analysis of this full-length transcript (ACUA027268) revealed that it has four coiled-coils features and one conserved GRIP domain at the 3′ end of the sequence (Fig. 2b; Table 1). Multiple sequence alignment and phylogenetic analysis revealed a high degree of sequence conservation within the mosquito and other insect species (Fig. 2c). RT-PCR analysis indicated that *Ac-qtc* was constitutively expressed in all the aquatic stages of development, except in the egg of the mosquitoes (Fig. 2d). This

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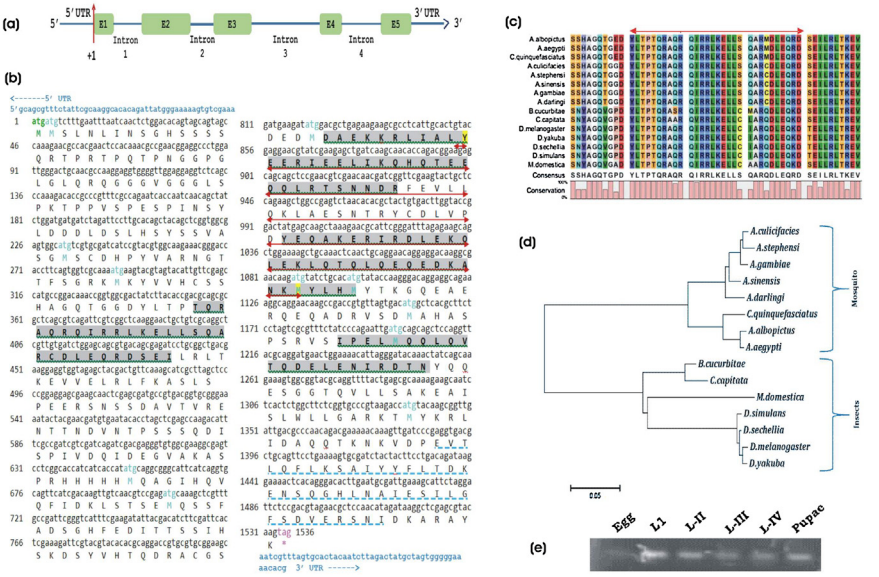

Fig. 2. Genomic and molecular characterization of *An. culicifacies quick-to-court* gene: (a) Schematic representation of the genomic architecture of the mosquito *Ac-qtc.* Five green coloured boxes (E1-E5) indicates the exons and +1 mark the transcription initiation site. (b) Gene organization and molecular features of full length *Ac-qtc*: The gene contains 1536 bp nucleotide, encoding 511 AA long peptides with four coiled-coils domains. Both 5′ and 3′-UTR regions are highlighted (Light blue) letters. The complete coding region of 511 amino acids starts from ATG/Methionine/green colour, ending with TAG/Red/*. The different features are highlighted with different colour code, viz. Coiled-coil domains (grey colour and green underlined), Pre-folding domain (Red arrow) and GRIP-domain (sky blue dotted line). (c) Multiple sequence alignment of selected coiled coil domain (marked with green arrow) and (d) phylogenetic relationship of *Ac-qtc* with other mosquito species and *Drosophila* (e) Developmental expression of *Ac-qtc* in *An. culicifacies* by RT-PCR.

suggested that the quick-to-court protein is evolutionary conserved with the common function in the regulation of insects behavioural responses.

Table 1. Molecular features of predicted domain in the mosquito Ac-qtc gene.

SI. No.	Feature Type	Start Site	End Site
1.	Coiled-coils (Ncoils)	118	146
2.	Coiled-coils (Ncoils)	275	310
3.	Coiled-coils (Ncoils)	332	367
4.	Coiled-coils (Ncoils)	396	417
5.	Prefolding Domain	285	363
6.	GRIP domain	463	504

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3.2. *Ac-qtc* abundantly expresses in the olfactory and brain tissues of adult mosquitoes

Sex and tissue-specific transcriptional profiling of *Ac-qtc* in the naive mosquitoes revealed the *qtc* gene expression in the olfactory tissue, brain and reproductive organs of both the sexes (Fig. 3). The previous study in *D. melanogaster* also demonstrates that *Dm-qtc* is expressed in the olfactory organs, central nervous system of both the sexes and male reproductive tract [18]. Interestingly, a relatively higher abundance of *qtc* gene in the olfactory tissue of male *An. culicifacies* indicated its possible involvement in the regulation of mosquito mating behaviour.

3.3. The sex-specific and age-dependent expression may regulate olfactory system maturation

Unlike *Drosophila*, unavailability of the proper molecular marker restricted our understanding of the complex mating biology in mosquitoes. In particular, studies on *An. culicifacies* mating behaviour are too limited [28]. However, our identification of the *quick-to-court* transcript from the blood fed olfactory tissue transcriptome data depicts that *Ac-qtc* may regulate the key molecular factors driving sex-specific behavioural modulation in *An. culicifacies.*

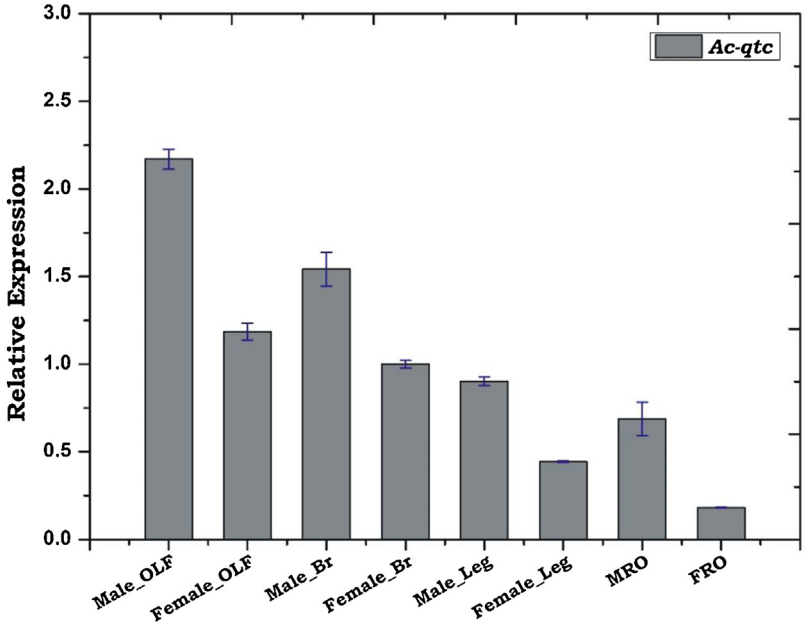

Fig. 3. Tissue and sex specific relative expression analysis of *Ac-qtc* in the adult mosquito. Male OLF: Male olfactory tissue (Antennae, maxillary palp and proboscis); Female OLF: female olfactory tissue; Male-Br: Male brain; Female-Br: female brain; Male-leg; Female-leg; MRO: Male reproductive organ; FRO: Female reproductive organ.

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To test this hypothesis, we first examined the sex-specific relative expression of a pool of four transcripts including *qtc*, that were identified from the ongoing olfactory tissue transcriptomic study of blood fed adult female mosquito (Fig. 4a). Our data indicated that male olfactory system matures faster than female olfactory system even if they are at the same age. Next, we monitored the age-dependent transcriptional regulation of *Ac-qtc* in virgin male and female mosquitoes. Irrespective of the mosquito sexes, it showed age-dependent enrichment of *Ac-qtc* at the highest level (~6- fold up-regulated/$p \leq 0.004$) on the 5th day, when compared to 1-day old virgin mosquitoes (Fig. 4b, c), followed by at least 2-fold down-regulation ($p \leq 0.0172$) on the 7th day. Interestingly after 7th-day *Ac-qtc* expression switched to up-regulation in male mosquitoes but it remained constant after 5th day in case of female mosquitoes. A similar pattern of *Ac-qtc* gene expression was also observed in the possibly mated male mosquitoes (Fig. 4d), which were collected from a mixed cage containing an equal number of male and female mosquitoes.

Although, it is yet unclear that how environmental guided non-genetic and/or genetic factors regulate the complex sexual behavioural events. But, our observations indicated that once male mosquitoes achieved the specific age of adulteration, the natural dysregulation of *Ac-qtc* by unknown mechanism may

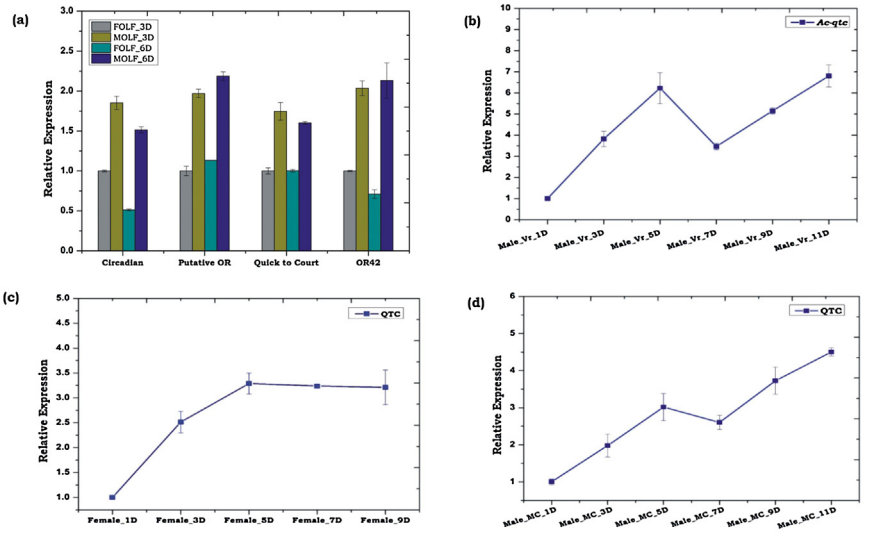

Fig. 4. Sex specific and age dependent transcriptional response of *Ac-qtc* transcripts in the olfactory tissue of *An. culicifacies.* (a) Expression analysis of four transcripts viz. *circadian*, *putative olfactory receptor* (putative OR), *quick-to-court* and *olfactory receptor 42* (OR42); FOLF: Female Olfactory; MOLF: Male Olfactory; (b-c) Age dependent transcriptional profiling of *Ac-qtc* in the virgin male and female mosquitoes (c): Male-Vr-1D: Male virgin mosquito of 1 Day old; Female-Vr-1D: Female virgin mosquito of 1 Day old. (d) Age dependent relative expression analysis of *Ac-qtc* transcript in the mated mosquito. Male-MC-1D: Male mosquito of 1 day old, collected from mixed cage (MC) containing equal number of male and female mosquitoes.

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promote the courtship behaviour (see next paragraph). These results also corroborate with the previous findings in *Drosophila* where a mutation in *Dm-qtc* gene causes accelerated male-male courtship behaviour [18]. A recent study by *Houot et al*. also suggested that the *qtc* and *shaker* genes, which are abundantly expressed in the neuro-olfactory system of *Drosophila*, decrease the ability to discriminate between the sex targets [28, 29, 30, 31]. This is probably due to the declined perception to wild type female pheromone [28, 29, 30, 31].

3.4. Natural dysregulation of *Ac-qtc* may promote mating success

An alternative interpretation, of the above argument, could be that a significant downregulation ($p \leq 0.0172$) of *Ac-qtc* in the 5–7 day old virgin adult male mosquitoes may be crucial for the auto-activation of courtship behaviour in case of *An. culicifacies* mosquitoes. In the lack of any molecular marker for mating behaviour studies, we attempted to trace the possible functional correlation of male *Ac-qtc* in the mating success i.e. insemination event completion in the laboratory-reared mosquitoes. However, our initial experiments of *Ac-qtc* gene silencing using purified dsRNA injection in the thorax of male mosquito's remains unsuccessful, primarily due to a high mortality rate of these mosquitoes (data not shown).

Available literature suggests that in most *Anopheline* mosquitoes, the mating behavioural activities commenced by the onset of sunset, usually at 1700 h which may continue till 2000 h [29, 32, 33]. We hypothesize that the transcriptional modulation of *Ac-qtc* in response to dawn/dusk cycle must have a functional correlation with the mating success, especially insemination events where adult females receive and store the sperms in their spermathecae delivered by the male during copulation [34, 35]. For experimental verification of this idea, we first identified two sperm-specific transcripts from the draft genome of *An. culicifacies*, using (*ams* and *mts*) as query sequences, previously characterized from *An. gambiae* [26]. To validate sperm specificity, the primers designed against *Ac-ams* (ACUA010089) and *Ac-mts* (ACUA014389) were tested by RT-PCR in the male accessory glands (MAG) and spermathecae (SPT) collected from laboratory reared 3–4 day old virgin male and female mosquitoes, respectively (Fig. 5a). A non-specific poor amplification was visible in case of *Ac-ams* in virgin female mosquito spermathecae. This was verified by incorrect melting curve signal that appeared in Real-Time PCR data (Supplemental Fig. S1). To trace the possible functional correlation of *Ac-qtc* in mating success, we collected olfactory and reproductive tissues two hours prior or later onset of the sunset as described in methodology section (See experimental design Fig. 1b). We then analysed and compared sex-specific regulation of *Ac-qtc* in the olfactory tissue, and sperm-specific *Ac-ams/Ac-mts* genes in the male and female reproductive organs.

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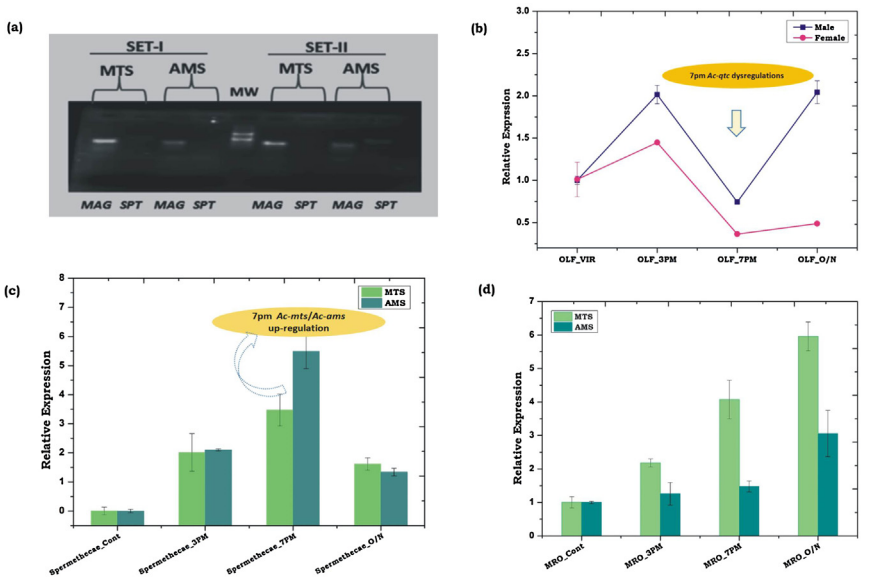

Fig. 5. (a) RT-PCR based expression validation of *Ac-ams* and *Ac-mts* in MAG (Male Accessory Gland) and SPT (Spermathecae). (b) Sex-specific transcriptional profiling of *Ac-qtc* at different circadian time in the olfactory tissues of the mosquito *An. culicifacies*; (c) Transcriptional response of *Ac-mts* and *Ac-ams* in the spermathecae of *An. culicifacies* at different circadian time. (d) Transcriptional response of *Ac-mts* and *Ac-ams* in male reproductive organ (MRO) at different circadian time. OLF: Olfactory; VIR: Virgin; Cont: Control; O/N: Overnight.

When compared to the virgin counterpart, significant down-regulation of *Ac-qtc* was observed in the olfactory system in both the sexes at 1900 h (Fig. 5b). This indicated that the lower expression of *Ac-qtc* may favour the increased mating frequency and active courtship engagement. A significant modulation of *Ac-ams/Ac-mts* expression in the mated female spermathecae (Fig. 5c) as well as male reproductive organs (Fig. 5d) supports the idea that a natural dysregulation of *Ac-qtc* in the late evening i.e. 1900 h may favour the copulation process by facilitating the release of unknown sex driving factors for successful insemination in the copulating couples. However, the exact molecular mechanism of qtc protein mediated regulation of insect's mating events is still unknown.

3.5. *Ac-qtc* regulation is independent of the nutritional status of the mosquitoes

Though feeding and mating are two mutually exclusive behavioural properties of any biological system. But those behaviours are dependent on each other to some extent at least for insects to facilitate the reproductive success and consequently their survival. The molecular basis of the sex-specific regulation of these conflicting behavioural demands (feeding *vs.* mating) remains largely unknown. Current studies in *Drosophila* suggested that food odour and sex-specific pheromone signals in the neuro-olfactory system work collaboratively to drive both the meal and/or mate

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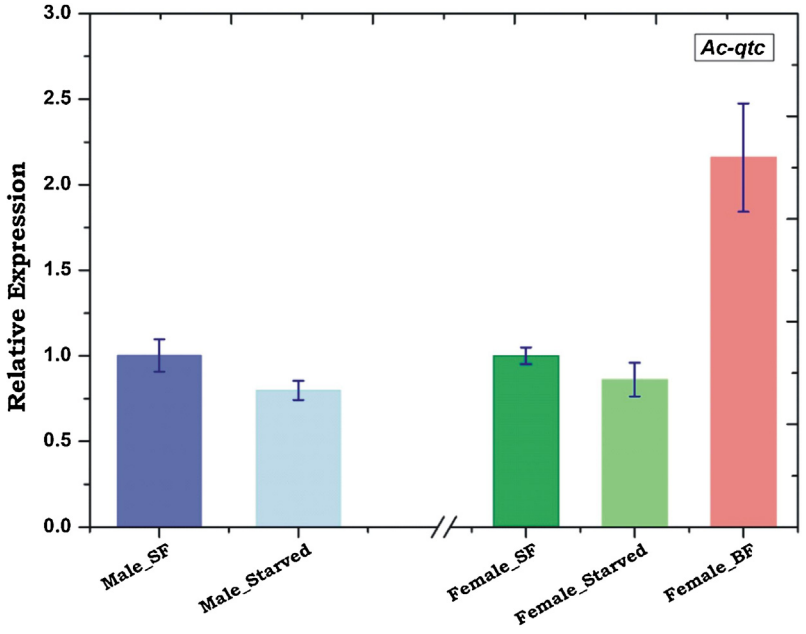

Fig. 6. Effect of starvation on *Ac-qtc* expression: both male and female mosquitoes were kept starved for 16–18 h and the olfactory tissues were collected for *qtc* expression study. Additionally blood meal was provided to female mosquitoes after starvation. SF: Sugar Fed; BF: Blood Fed.

attraction [31]. Thus to test whether *Ac-qtc* has any sex-specific relation to the nutritional status of naïve mosquitoes, we examined and compared the relative expression of *Ac-qtc* transcript in starved and sugar fed mosquitoes in both the sexes. To perform this experiment, we collected olfactory tissue from 5-6-day old sugar fed and 16–18 h starved mosquitoes. Relative gene expression analysis indicated that starvation did not affect the abundance of *qtc* transcript, but showed a two-fold up-regulation ($p \leq 0.03$) in response to immediate i.e. 30 min − 1 h post blood feeding (Fig. 6). Together these data suggested that *Ac-qtc* may not be essential for regulating mosquito sugar feeding behaviour but it may have an important role in the regulation of host-seeking/blood feeding behaviour in the adult female mosquitoes.

3.6. Blood meal alters expression of *Ac-qtc* in adult female mosquito

To further evaluate *Ac-qtc* role in response to blood feeding behaviour of female mosquitoes, we performed a blood meal time series experiment as described earlier [3]. We collected olfactory tissue from *An. culicifacies* mosquito depending on their age and blood feeding status. *Ac-qtc* expression analysis revealed increased abundance till 5th day, but significant (2.5 fold/$p \leq 0.03$) downregulation on 7th day in the naive unfed adult female mosquitoes. This pattern is similar to the adult male mosquitoes (Fig. 7), which may probably to achieve the optimal courtship behavioural success in both the sexes.

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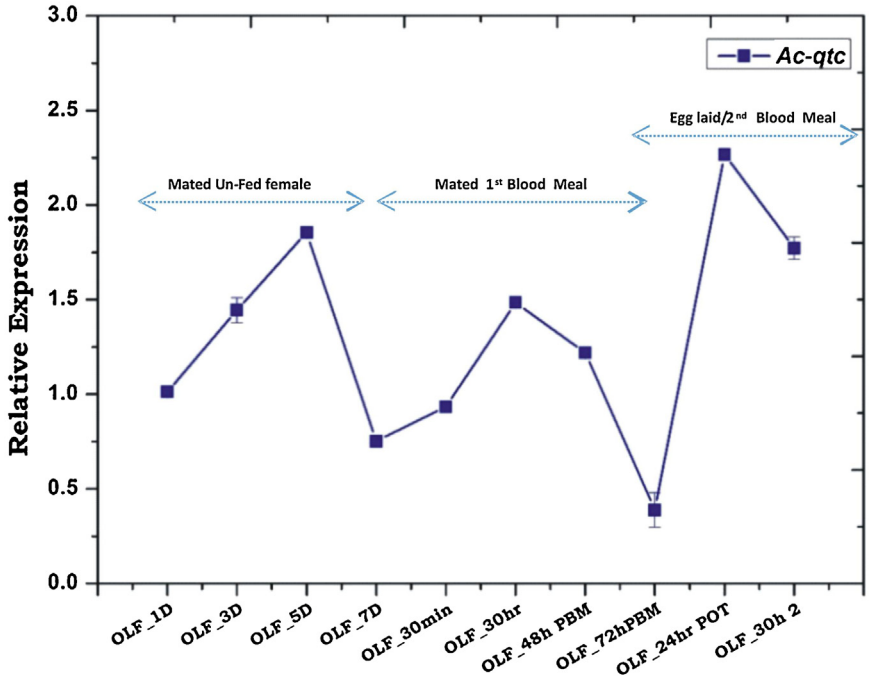

Fig. 7. Transcriptional behaviour of *Ac-qtc* in two consecutive blood meal follow up. OLF-1D – OLF-7D: Olfactory tissue from 1Day –7 Day old female; OLF–30 min: Olfactory tissue collected from 30 min post blood fed mosquito; OLF-30 h: Olfactory tissue collected from 30 h post blood fed mosquito; OLF-48 h: Olfactory tissue collected from 48 h of post blood fed mosquito; OLF-72 h: Olfactory tissue collected from 72 h post blood fed mosquito; OLF-24 h POT: Olfactory tissue collected from 24 h of post oviposition of mosquito; OLF-30 h 2: Olfactory tissue collected from 30 h of 2nd blood meal. PBM: Post Blood Meal.

Although it is not clear whether the first blood meal transiently and/or completely pauses re-mating events, but a consistent up-regulation of *Ac-qtc* just after blood feeding (within 30 min) till 30 h post blood meal (Fig. 7) indirectly suggested that adult female mosquito may not seek any courtship event at least for the first 30 h post blood meal. Furthermore, the continuous sharp downregulation (~4 fold) of *Ac-qtc* till 72 h post first blood meal still remains questionable. It is unclear, whether *Ac-qtc* promotes host-seeking behaviour for second blood meal and/or it initiates mate partner finding. Significant up-regulation (~1.3 fold; Fig. 7b) of *Ac-qtc* after oviposition and prior to second blood meal also support our hypothesis. Second blood meal again rapidly downregulated the *Ac-qtc* expression, when examined 30 h post blood fed in the olfactory tissue. Together these data indicate that *Ac-qtc* may have a unique role in driving dual mode of behavioural responses possibly to meet the conflicting demand of sexual mate partner and/or finding a suitable vertebrate host for blood feeding.

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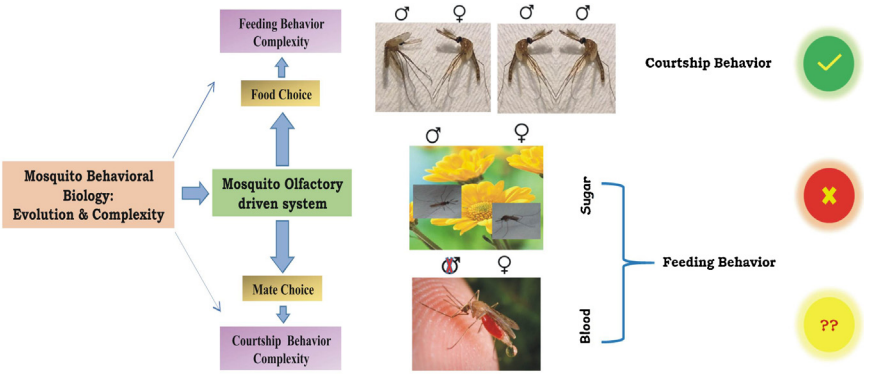

Fig. 8. Proposed hypothesis for the possible function of *quick-to-court* gene in mosquito. Photo credit to James Gathany for the blood fed mosquito picture.

4. Conclusion

Understanding the sex-specific molecular genetics of mosquito's behavioural biology is more complex. This is partly due to unique nature of blood feeding evolution and adaptation in the adult female mosquitoes, and limitation of generating mutants. Through comprehensive molecular approach, we examined the sex-specific transcriptional regulation of a unique transcript *Ac-qtc*, and predicted its possible role with 'food choice' and/or 'mate choice' behavioural performance (Fig. 8). Our data provides the first molecular evidence that *Ac-qtc* proteins may have a dual mode of action in the regulation of a cluster of mosquito olfactory genes that are linked to mating success and/or blood feeding in adult female mosquitoes. We believe these findings may guide to uncover the functional nature of *Ac-qtc* controlling complex mating and/or blood feeding behaviour in mosquitoes.

Declarations

Author contribution statement

Tanwee Das De: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Punita Sharma, Charu Rawal, Seena Kumari, Sanjay Tavetiya, Jyoti Yadav: Performed the experiments.

Yasha Hasija: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Rajnikant Dixit: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

Data associated with this study has been deposited at Genbank under the accession number KX575650.

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