Immune Responses of *Anopheles stephensi* and *Anopheles culicifacies* against Malaria Parasite Development

THESIS

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CERTIFICATE

This is to certify that the thesis entitled **"Immune Responses of *Anopheles stephensi* and *Anopheles culicifacies* against Malaria Parasite Development"** submitted by **Rini Dhawan** ID No **2011PHXF404P** for award of Ph.D. degree of the institute embodies original work done by her under my supervision.

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ABSTRACT

Mosquito fauna is rich in the tropical climate with numerous and diverse breeding resources and responsible of transmitting several diseases. Malaria is transmitted through *Anopheles* mosquitoes. In India, out of 58 known species of *Anopheles,* only six species are the major malaria vectors distributed in different geographical region. *Anopheles culicifacies s.l.* is the vector of rural malaria in the country whereas *An. stephensi* is the well-known vector species of urban malaria. India is experiencing rapid ecological changes owing to population explosion, urbanization, development projects, deforestation and human migration affecting mosquito ecology and disease transmission. These many factors are enough to seriously impede the country's malaria control efforts and therefore are subject to urgent attention. Transmission blocking can prove to be a novel approach for solving the purpose. Amongst various approaches such as mechanisms of vector resistance or the release of genetically modified or non-infectious mosquitoes to block transmission, utilization of mosquito immunity to develop transmission blocking vaccine is the most appealing one. It is now well known that mosquito possess strong innate immunity which is able to kill different invading pathogens. However, different mosquito species responds differently to the same pathogen species which further complicates the situation. Therefore, in this thesis important immune component of two Indian malaria vector, *Anopheles stephensi* and *Anopheles culicifacies* has been studied, individually.

Apolipophorin III (ApoLp-III) is a well-known hemolymph protein having a functional role in lipid transport and immune response of insects. Molecular and Functional characterization of *An. stephensi* Apolipophorin-III (AsApoLp-III) is reported in this study. The gene consists of 679 nucleotides arranged into two exons of 45bp and 540bp that gives an ORF encoding 194 amino acid residues. Secondary structure analysis shows that 89% of the amino acid residues are arranged in alpha helix with no beta strands in the peptide typical of protein involved in lipid-protein stable interaction. Phylogenetic analysis shows the divergence of ApoLp-III of mosquitoes (Order Diptera) from their counterparts in moths (Order: Lepidoptera & Homoptera). Also, it reveals close relatedness of AsApoLp-III from Apolp-III of *An. gambiae.* AsApoLp-III expression was detected in all the life stages of *An. stephensi* with the maximum expression occurring at larval life form. AsApoLp-III mRNA expression is strongly induced in *Plasmodium berghei* infected mosquito midguts suggesting its crucial role in parasite development. AsApoLp-III silencing decreased *P. berghei* oocysts numbers by 7.7 fold against controls. These effects might be due to the interruption of AsApoLp-III mediated lipid delivery to the developing

oocysts. In addition, nitric oxide synthase (NOS), an antiplasmodial gene, is also highly induced in AsApoLp-III silenced midguts suggesting that this gene acts like an agonist and protects *Plasmodium* against the mosquito immunity.

Signal Transducer and Activator of Transcription (STAT) is another immune gene regulates malaria parasite development in *An.gambiae* and *An.stephensi* through induction of NOS. Here we used laboratory colonized *An. culicifacies* to identify the STAT pathway genes *viz.* AcSTAT-A, AcSOCS and AcPIAS that were cloned and sequenced using specific primers. Phylogenetic analysis of these STAT pathway genes confines them to the cluster belonging to Anophelines thus separating them from their counterparts in insect belonging to other dipterans. The STAT pathway genes were present in all the stages of mosquito development with prominence at initial egg stages. Also, the q-PCR analysis hints towards the male biasness of these genes. The effect on expression of these genes in *An. culicifacies* mosquito 24 hour after *P. berghei* infection was investigated. Quantitative PCR analysis revealed that AcSTAT-A mRNA levels were not affected upon *P. berghei* infection. In case of AcSOCS, near about four times higher expression was observed in the infected midguts whereas AcPIAS expression was also found to be significantly high in midgut compared to blood-fed control. This indicated that STAT being a transcription factors, activates the pathway during ookinete invasion and in parallel the suppressors (SOCS and PIAS) of this pathway were induced to counter balance the immune reactions.

The role of Reactive oxygen species (ROS) in immunity was analyzed in *An. gambiae* and *An. stephensi* species. However, there is no report of ROS in *An. culicifacies* immunity. Therefore, in fourth section of thesis, we identified and characterized ROS detoxifying enzyme catalase and superoxide dismutase (SOD) in *An. culicifacies*. Phylogenetic analysis shows their close relatedness with genes present in *An. stephensi* while separating them from *Aedeine* and *Drosophilidae*. These detoxifying genes were expressed in all the stages of mosquito development. The upregulated expression of AcCatalase during pupal stage clues their role in metamorphosis which is the major event in pupae. The expression profile for AcSOD and AcCatalase was checked in response to *P. berghei* infection. It was seen that AcSOD in the midgut tissue 24 hour post infection shows an induction, though not significant but a sharp downfall of 6 fold was analyzed in infected carcass compared to control. The reduced expression of Cu-ZnSOD in *An. culicifacies* at this time of high metabolic activity would increase intracellular levels of free radicals in the mosquito body that help in the clearance of malarial parasite load. On the other hand, AcCatalase mRNA levels were induced in the infected midgut and carcass as compared to control. The

mosquito tends to lower H_2O_2 generated as an immune response to normal levels in their body, a probable step to neutralize increased production by the activated immune system. This showed that there occurs an intricate interplay between the ROS production and expression of detoxifying enzymes to overcome the fray against parasite as well as to maintain homeostasis within mosquito species. The overall study suggested that these immune molecules are present in all *Anopheles* species; however host-parasite interaction varies from one species to other species. Therefore, detail study of Indian *Anopheles* species are necessary to control malaria development.

Dedicated to My Beautiful Family
& Beloved Husband

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Rini Dhawan,

BITS Pilani

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ABBREVIATIONS

aa	Amino acid
Ae	*Aedes aegypti*
An.	*Anopheles*
Ag	*Anopheles gambiae*
AGCC	*Anopheles* Genome Cluster Committee
AMP	Anti-microbial peptide
ApoLp	ApoLipophorin
As	*Anopheles stephensi*
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
cDNA	Complementary DNA
CE	Cholesteryl ester
ct	Threshold cycle number in a PCR
Δct	Difference in ct values
DAG	Diacyl glycerol
DDT	Dichloro-diphenyl-trichloroethane
dsRNA	Double stranded RNA
HDLp	High density Lipoprotein
JAK	Janus Kinase
LDLp	Low density Lipoprotein
Lp	Lipophorin
LPS	Lipopolysaccharides
MEGA	Molecular Evolutionary Genetics Analysis
NCBI	National Center for Biotechnology Information
NJ	Neighbour Joining
NO	Nitric Oxide
NOS	Nitric Oxide Synthetase
P.	*Plasmodium*
Pb	*Plasmodium berghei*
PCR	Polymerase Chain Reaction
Pf	*Plasmodium falciparum*
PIAS	Protein Inhibitor of Activated STAT

PO	Phenol Oxidase
PRR	Pattern Recognition Receptor molecule
PTGS	Post-transcriptional gene silencing
qPCR	quantitative real time PCR
RISC	RNA-induced silencing complex
RNAi	RNA interference
S7	Ribosomal protein Sub-unit 7
siRNA	Small interfering RNA
SOCS	Suppressor of Cytokine signaling
SOD	Superoxide Dismutase
STAT	Signal Transducer and Activator of Transcription
TEP-1	Thio-ester like Protein-1
WHO	World Health Organization

Molecular and Functional Characterization of Apolipophorin-III in Anopheles stephensi

In the chapter, molecular and functional characterization of Apolipophorin-III gene in major Indian Malaria vector, *An. stephensi* has been explained. Here, its role during *Plasmodium* infection within the mosquito has been analyzed in details. AsApoLp-III mRNA expression is strongly induced in *Plasmodium berghei* infected mosquito midguts suggesting its crucial role in parasite development. AsApoLp-III silencing decreased *P. berghei* oocysts numbers by 7.7 fold against controls. These effects might be due to the interruption of AsApoLp-III mediated lipid delivery to the developing oocysts. In addition, nitric oxide synthase (NOS), an antiplasmodial gene, is also highly induced in AsApoLp-III silenced midguts suggesting that this gene acts like an agonist and protects *Plasmodium* against the mosquito immunity.

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Chapter 4

4.1 Introduction

Apolipophorin-III (ApoLp-III) is an amphipathic insect hemolymph protein that binds hydrophobically with lipoprotein surfaces that allows lipid transport in aqueous media (Van der Horst et al., 1993; Blacklock and Ryan, 1994). Homologous to a mammalian lipoprotein Apolipoprotein E, ApoLp-III facilitate loading of diacylglycerol present in insect fat body onto lipophorin with the involvement of adipokinetic hormones (Feingold et al., 1995).

Recently, ApoLp-III was documented as an immune-stimulating protein (Kim et al., 2004; Weisner et al., 1997; Niere et al., 1999; Halwani et al., 2001; Whitten et al., 2004) mediating both humoral and cellular immune responses. ApoLp-III binds to a varied range of immune elicitors including lipoteichoic acid (LTA), present in the cell wall of Gram-positive bacteria (Halwani and Dunphy, 1999; Halwani et al., 2001), lipopolysaccharide (LPS) layer of Gram-negative bacteria (Dunphy and Halwani, 1997; Kato et al., 1994) and beta 1, 3 glucan of fungal cell walls (Whitten et al., 2004). These binding properties impart ApoLp-III to have role pertaining to pathogen recognition receptor (PRR).

ApoLp-III also increases antibacterial activity (Park et al., 2005; Weisner et al., 1997; Niere et al., 1999; Whitten et al., 2004) to be involved in activation of the prophenoloxidase cascade (Kim et al., 2004), and to participate in cellular immune responses by increasing the phagocytic activity of isolated hemocytes in vitro (Weisner et al., 1997).

ApoLp-III is known to stimulate encapsulation, a hemolymph immune reaction that occurs in response to the systemic introduction of foreign, non-self substances that are too large to be phagocytized by a single hemolymph cell (Zdybicka-Barabas and Cytrynska, 2013). Both the lipid-associated ApoLp-III, and the lipid-free ApoLp-III enhanced the *in vitro* cellular encapsulation of curdulan–toyopearl beads and DEAE-Sepharose by washed hemocytes in a dose-dependent manner (Whitten et al., 2004).

Besides, ApoLp-III is confirmed to have a role in programmed cell death of muscle cells and neurons in *Manduca sexta* (Sun et al., 1995) and also seen to have role in hemagglutination in *Galleria mellonella* (Iimura et al., 1998). Moreover, ApoLp-III was detected among specific RNA-binding proteins, suggesting its role in extracellular RNA-mediated defense response (Altincicek al., 2008).

Taken together, ApoLp-III lucratively establishes itself as a multifunctional hemolymph protein involved in both humoral and cellular immune responses in several species of insects, such as *Heliothis virescens* (Chung and Ourth, 2002), *Hyphantria cunea* (Kim et al., 2004), *Anopheles gambiae* (Gupta et al., 2010), *Thitarodes pui* (Sun et al., 2012), and *Tribolium castaneum* (Contreras et al., 2013). However, not much research has been done on this immune molecule in mosquitoes except *An. gambiae.*

In the current chapter, molecular and functional characterization of Apolipophorin-III gene in major Indian malaria vector, *An. stephensi* has been done. Here, its role during *Plasmodium* vulnerable stages within the mosquito has been analyzed in details. This also gives an idea of the probable mechanism through which the immune system restricts the infection between particular strains of mosquitoes and specific parasites strain.

4.2 Results and Discussion

4.2.1 Cloning and Sequence analysis of *An. stephensi* ApoLp-III

The *An. gambiae* ApoLp-III cDNA sequence was selected as query to identify a putative AsApoLp-III cDNA from the *An. stephensi* genome database using the NCBI basic local alignment search tool (BLAST). Putative AsApoLp-III (as discussed in Chapter 3) from the available contig (No: 120 KE388890.1) was identified. To clone and confirm the predicted cDNA of *An. stephensi* ApoLp-III gene, primers were designed and PCR was performed the using midgut cDNA as template. The primers (5' to 3') used were as follow: ApoLp-III Fw: AGCCCAATTTCTTCCAGACC and ApoLp-III Rev: CGGTTGCTTCAGCTCGTT (Fig. 4.1) which yielded product size of 482bp keeping annealing temperature of 55°C.

Another set of primer was designed (Fig. 4.1, shown in broken arrows) for checking the silencing of the ApoLp-III gene (will be discussed in section 4.2.4.1).

```
  1 atgGCGAAGCTTCTGTGCCTTATcctggccctgtgtgccgttcagggaactctgggattc
      >>>>>>>>>>>>>>>>>>>

 61 gttcgccgtgacgcccccgctgcaccagcggaggAGCCCAATTTCTTCCAGACCatcatg
                                     <<<<<<<<<<<<<<<<<<<

121 aacatcaaggacaAGATTGAGGTGGCATTCGAGgaaacgcagcagaacgttctgaagtct
                <<<<<<<<<<<<<<<<<<<

181 ctcgggttccagtcgaacgaggaggtcgtccagacgatccagcaaaacaccaaccagtacgtg

244 gatcggctgcgatcagtgcagggcgtgcttgacgaggaggtgaagaagcattcggacatcttc

307 gagcccatcgtgaaggatctgaaggttaagctggaccaaaccactgccaccctgtccgagcag

370 aacccggaagttgtgcagaaagcgaaggagtaccaggagcagctgacgaccaacgtacagtcc

433 ctggtggccgaggctcagaagacggtcgaaaagctaaaggccgatacgcgcgtacagtccgag

496 gagatgcagaaggcattgaagatgttgtacgactcgactttcgaagtcttcaccaagacggcc

559 AACGAGCTGAAGCAACCGaagaactaa
    <<<<<<<<<<<<<<<<<<<
```

Fig. 4.1 Predicted cDNA sequence of AsApoLp-III: Primer set shown in bold line were used for cloning of gene. The primer set shown in arrows were used to check silencing of Apo-Lp-III gene.

To confirm the putative AsApoLp-III sequence, PCR was carried out using above set primers, the obtained 482 bp fragment was cloned into PCR- TOPO TA cloning vector (Invitrogen) and the positive colony was further confirmed through sequencing (Fig. 4.2). The sequence obtained was matched with the putative AsApoLp-III (as mentioned in Chapter 3) and it was found to be identical to the prediction done.

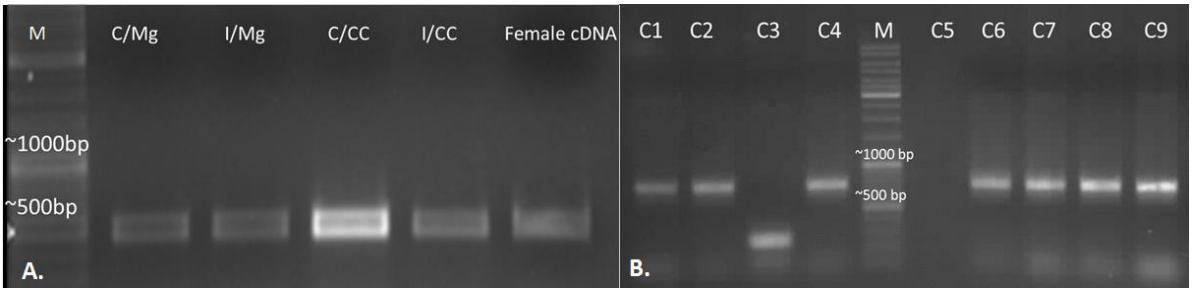

Fig. 4.2(A) PCR products amplified with respective cDNA using primer set (lanes 2, 3, 4, 5, 6 expected size 482bp). (B) Verification of AsApoLp-III clones with M13 primers (lanes 1, 2, 4, 6, 7, 8, 9 expected size 702bp) using nine bacterial colonies as templates.

BLAST searches further confirmed that AsApo-Lp-III shares similarities with Apolipophorin from other organisms. Not only is it more than 80% similar with mosquito

ApoLp-III genes but also shares considerable similarity with Apolipophorins from sequence of other reported insects. This was the foremost report of Apolipophorin from *An. stephensi* and its gene sequence has been submitted in NCBI GenBank database (Accession No: KU051523).

4.2.2 AsApoLp-III expression in different developmental stages of *An. stephensi*

Despite being a multi-functional protein, its primary function includes transport of lipid and egg production in many insects at varying stages (Telang et al., 2013). Therefore to elucidate its probable role in *An. stephensi* in different developmental stages, quantitative real time PCR (qPCR) analysis was done. It indicates that AsApoLp-III expression was detected in all the life stages of *An. stephensi* with the maximum expression occurring at larval life form (Fig. 4.3).

Anautogenous mosquitoes are obligatory blood feeders that utilize the nutrients in blood to nourish their eggs. Ovaries of newly emerged anautogenous mosquitoes are arrested at an early stage of development. With intake of blood meal, a complex hormonal cascade is followed that result in the continuation of ovarian development and ends in the maturation of a batch of eggs. The reason for elevated levels of ApoLp-III at larval stages can be attributed to the fact that larval nutrition significantly affects the endocrinology of egg development in mosquitoes. Apolipophorin-III is very well known to play a crucial role in vitellogenesis during egg development (Telang et al., 2006) such that this gene may be anticipated to be a part of teneral reserve which in turn is responsible for affecting important female reproductive processes, such as utilization of reserves, fecundity, longevity and blood meal consumption and utilization (Briegel, 1990a; Briegel, 1990b; Briegel et al., 2002; Naksathit et al., 1999a; Naksathit et al., 1999b; Takken et al., 1998;Zhou et al., 2004). Thus, both larval-derived teneral reserves and a blood meal in anautogenous females can serve as sources of yolk precursors and as stimuli for hormonal regulation of egg maturation.

There occurs a basal level of expression of ApoLp-III in pupal stage since mosquito at this stage do not feed and therefore function related to transport of lipids are not required. ApoLp-III seems to have minimal role during the metamorphic reactions prominent in the pupal stage. Presumably, mRNA level of ApoLp-III is more in female mosquito than in male pertaining to its role in lipid transport during egg production.

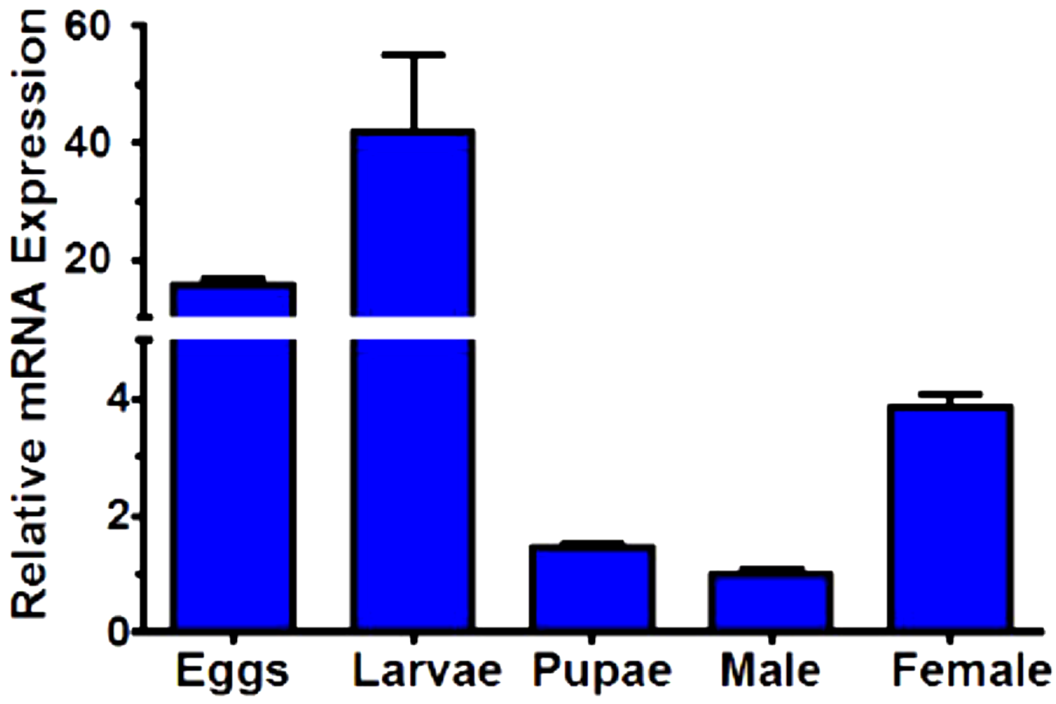

Fig. 4.3 AsApoLp-III Expression in different developmental stages of *An. stephensi*

4.2.3 Expression analysis of AsApoLp-III in response to *Plasmodium* infection

The *Plasmodium* cycle commences when female *Anopheles* mosquito ingests gametocytes-infected blood. Ingested gametocytes differentiate into macro- and micro-gametes in the mosquito midgut immediately after blood feeding. These motile micro (male) gametes emerge from erythrocytes through a process called exflagellation (Billker et al., 1997). This is followed by the fertilization of macrogamete which leads to the formation of non-motile zygote. During 15-20 h post fertilization, the zygote differentiates into motile ookinete and penetrates the non-membranous peritrophic matrix. This matrix surrounds the blood bolus and acts like a barrier between the gut epithelium and the bolus antigens (Kumar et al., 2010; Kajla et al., 2015b). *Plasmodium* invades mosquito gut cells and encounters the immunity, which limits their growth and development (Baton and Ranford-Cartwright 2005). It may be a general phenomenon of interaction of *Plasmodium* with vector *Anopheles* species and defined as a strategy for parasite survival in host (Vinetz et al., 2005; Vlachou et al., 2006). During this time a variety of immune reactions occur within the mosquito in response to *Plasmodium* that plays a fundamental role in deciding the fate of parasite.

To find out the role of AsApoLp-III in immunity, AsApoLp-III mRNA expression in the adult female midgut and carcass at varied time points after *P. berghei* infected blood feeding was investigated by qRT-PCR. Midgut as the first immune organ having to come across by *Plasmodium* holds special consideration but due to the cross talk of compartmentalization within mosquito, expression pattern of AsApoLp-III was also profiled for carcass (mosquito body except Midgut).

The time kinetics of the gene on *P. berghei* infection depicts its biphasic pattern of induction in the midgut having induced at 3 h followed by 18 and 24 h with relatively low expression at 6, 9 and 12 h. AsApoLp-III mRNA expression in midgut was significantly upregulated (~4 times) at 3h after infected blood feeding compared to the control as shown in (Fig. 4.4A). 3h time point represents the pre-ookinete time period in which gamete fertilization and zygote production takes place in the gut of infected mosquitoes. The significance of 24h time point corresponds to ookinete invasion of the midgut epithelium. Induction pattern at 24h on *P. berghei* infection is in accordance with *An. gambiae* where it was seen that the gene induces due to the presence of ookinetes, as it was not observed when mosquitoes fed on an infected mouse were later maintained at 28°C temperature, a temperature unfavorable for ookinete formation (Gupta et al., 2010). The up-regulation at critical time points of 3h and 24h post infection (*p value* <0.0001) indicates its decisive role in establishment of *Plasmodium* infection. Transcriptional response of a mosquito gene to a parasite during the stressful situation of infection is an indicator of it having a significant role.

It is already known that neutral lipids including DAGs and TAGs along with CE and cholesterol accumulate in gametocytes in infected RBC's. This substantial enrichment of neutral lipids in mature gametocytes supply major energy storage to fuel the sudden increase in protein and phospholipid biosynthesis required during gametogenesis and early zygote development in the insect host when there is limited access to certain lipid species (Tran et al., 2016). The reason of increased ApoLp-III mRNA levels in infected mosquitoes when compared to uninfected can be attributed to the significant increase in overall lipid upon parasite infection and gametocyte maturation. On the other hand, uninfected RBCs contain hardly any storage lipids.

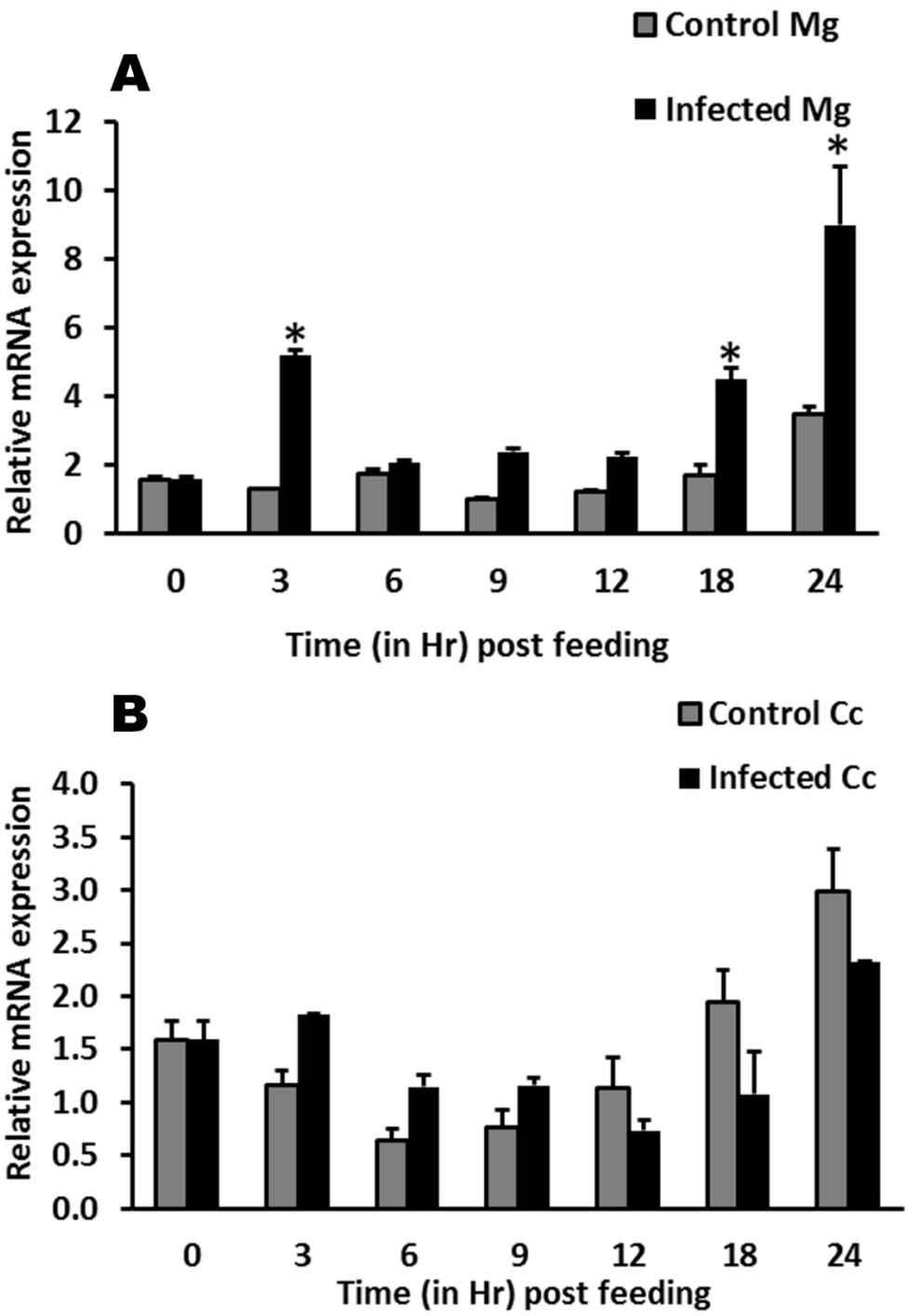

Fig. 4.4 Time kinetics of AsApoLp-III gene expression in *Plasmodium berghei* infected females. (A) Relative AsApoLp-III mRNA levels in female midguts fed on an uninfected (control) or *P. berghei*-infected mouse. Samples were collected at different time points after the blood feeding. (B) Same as (A) but carcass samples were collected from the same mosquitoes. Ribosomal S7 protein mRNA levels were used as internal loading reference.

Notably, the expression of ApoLp-III in midgut at the challenging period of parasite invasion along with previous reports on the role of ApoLp-III on *L. migratoria* and *G. mellonella* had shown that the protein can occur at different compartments and at different stages of the insect signifying its omnipotent role in insect survival during the time of infection (Chino and Yazawa, 1986; Wiesner et al., 1997; Zdybicka-Barabas and Cytryńska, 2012).

A similar expression pattern of AsApoLp-III was profiled for carcass (Mosquito body except Midgut) through qRT-PCR. Here, there was a slightly increased expression at 3, 6 and 9 h time point whereas the expression of AsApoLp-III reduced in infected carcass at further time points of 12, 18 and 24h as compared to control samples (Fig. 4.4B). Contrary to midgut response, ApoLp-III showed infection independent temporal induction in the carcass, consistent with the known origin of apolipoproteins in the fat body. Induction pattern on *P. berghei* infection in carcass displays that it is being expressed more in control sample than in infected at crucial time point of ookinete invasion. This directs the view AsApoLp-III gene to be more effective as immune molecule in the midgut tissue which is the foremost immune organ encountered by the parasites.

4.2.4 Functional characterization of AsApoLp-III through RNAi

RNA interference (RNAi) is a gene regulatory mechanism that controls the coding transcript level (mRNA) by either suppressing transcription (transcriptional gene silencing or TGS) or by activating a homology based mRNA degradation process (post-transcriptional gene silencing or PTGS) (Zamore et al., 2000, Tomari and Zamore, 2005). The process involves the production of double stranded RNAs (dsRNA) of target gene which is processed into 21–24 nucleotides RNA duplexes by the RNase III enzyme dicer and its homologs. These siRNAs are then incorporated into a multi-subunit endonuclease silencing complex called RNA-induced silencing complex (RISC). Argonaute proteins, the core catalytic components of RISC, use small interfering RNAs (siRNA) as a guide to recognize and degrade the complementary gene or mRNA (Hammond et al., 2000) (Fig.4.5).

In the past decade, RNAi approaches have been applied in numerous studies to untangle the genetic basis of the mosquito's susceptibility and the immune responses elicited by the parasite. A range of mosquito genes that react to *Plasmodium* infection have been identified

in latest years as studied in a number of reports **(**Cirimotich et al., 2010; Blandin et al., 2008; Marois et al., 2011**)**. These include genes that may either negatively influence *Plasmodium* development, i.e. antagonists, or have a beneficial effect on parasite development, i.e., agonists.

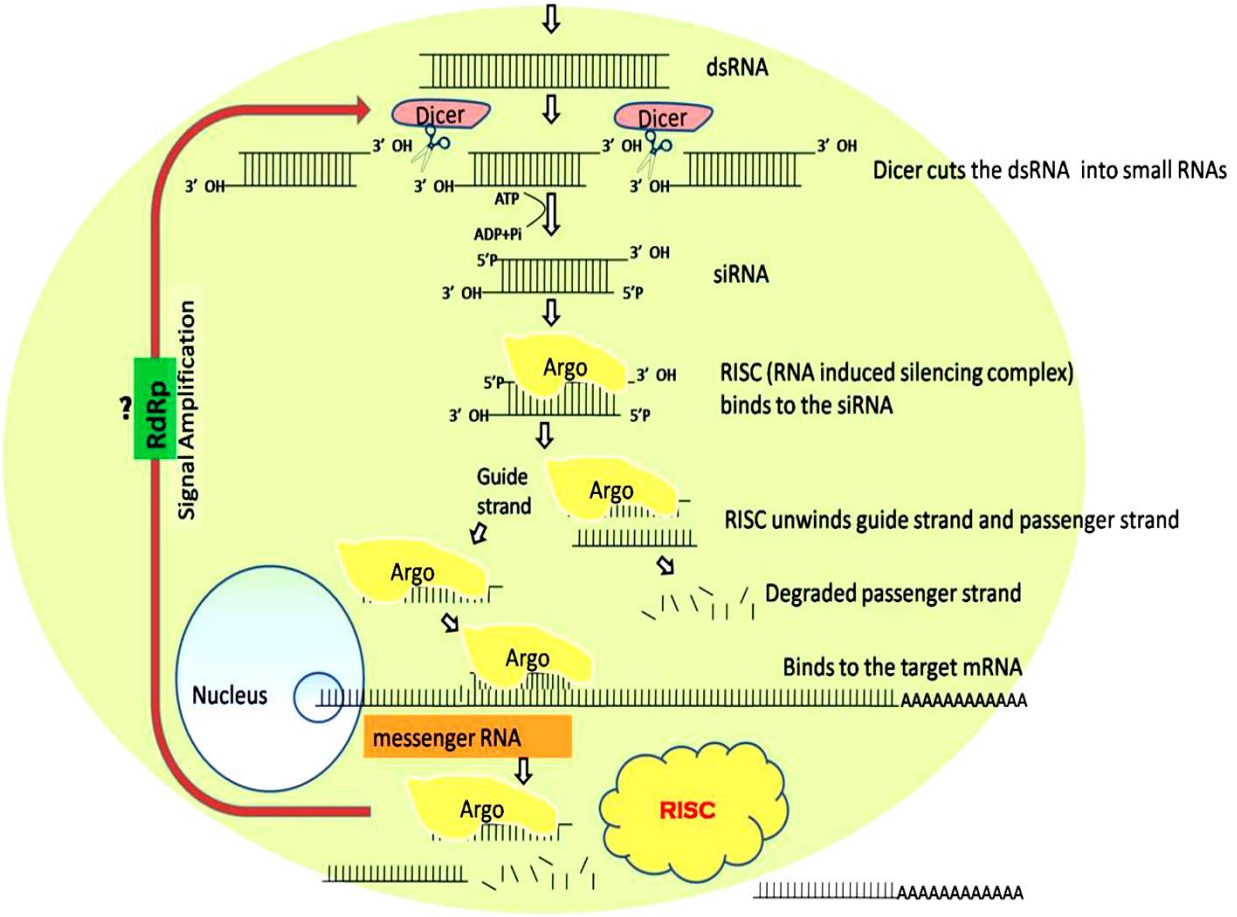

Fig. 4.5 Systematic illustration of gene silencing by RNAi inside the mosquito cells (Figure adapted from Kola et al., 2015).

To find out the role of ApoLp-III in *An. stephensi*, RNAi-mediated silencing approach for this gene was done in *An. stephensi*. For this dsRNA was prepared from the plasmid DNA of ApoLp-III gene cloned into TOPO-TA vector (Fig. 4.2). This TOPO-TA vector already has a T7 promoter at one end in its skeleton (Fig. 4.6), however, T7 promoter at the other end of fragment was incorporated by amplifying the cloned insert using primer: T7-M13R-CTCGAGTAATACGACTCACTATAGGGCAGGAAACAGCTATGAC. PCR amplification was carried out with 94°C for 5 min, 40 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and 72°C for 10 min using M13 Fwd and T7-M13 Rev primers.

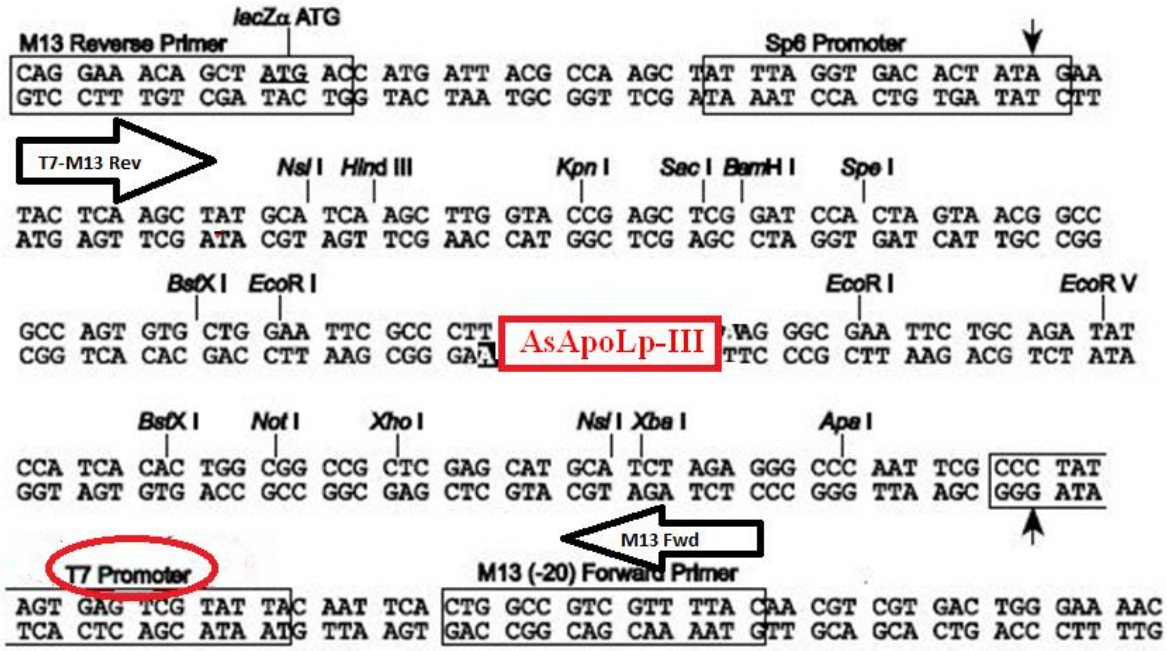

Fig. 4.6 The TOPO-TA vector map: This demonstrates the position of the 482 bp clone of AsApoLp-III (marked in red). T7 promoter at one end is already present (marked in oval). T7 promoter at the other end was incorporated through amplification with M13 Fwd and T7-M13 Rev primer (shown in arrow) of this clone.

Amplicons were extracted from gel with the QIAquick Gel Extraction Kit (cat no 28704, Qiagen, Valencia, CA, USA). Gel-purified amplicons tailed with T7 promoter sequences were used to synthesize dsRNAs with the MEGAscript kit (Cat No. AM1626, Ambion, Austin, TX). For this, eluted products were used for overnight transcription reaction (Fig. 4.7A). Transcribed products were used for annealing reaction at 75°C and further for ssRNA digestion (Fig. 4.7 B and C). dsRNA was further purified using a Microcon YM-100 filter (Millipore) and finally concentrated to 3 µg/µl in DNase and RNase free water. In similar fashion, dsRNA for LacZ gene was also prepared which was used as a control for the experiment. It is because the LacZ gene is absent in mosquito genome. A 218-bp fragment of the lacZ gene was amplified using the primers (5' to 3') Fwd-GAG TCAGTGAGCGAGGAAGC and Rev-TATCCGCTCACAATTCCACA and cloned into the pCRII-TOPO vector (Gupta et al., 2010). dsLacZ was prepared using same procedure as described above for dsApoLp-III. The final concentration of dsLacZ RNA was achieved to be 3µg/µl and stored in -20°C freezer for further use.

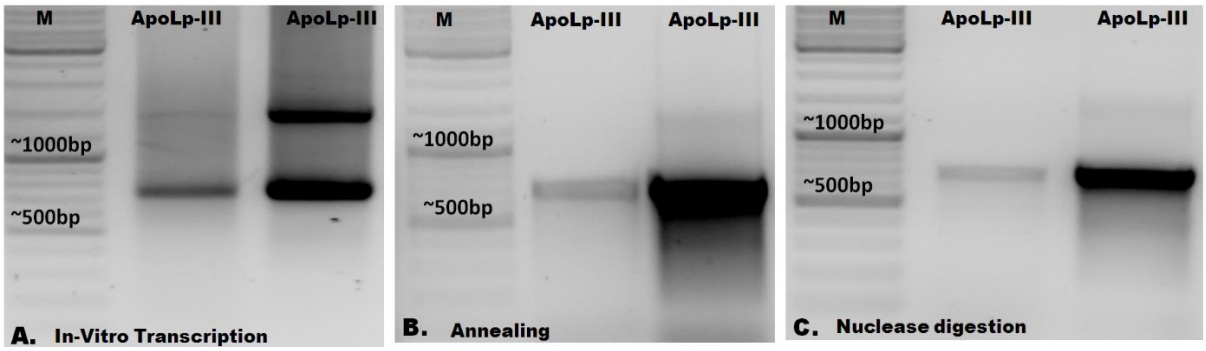

Fig. 4.7 Preparation of dsRNA for AsApoLp-III gene. AsApoLp-III gene was amplified and eluted PCR product cloned in to PCR-II TOPO TA vector. Addition of T7 overhangs in cloned plasmid template through M13 Fwd and T7 incorporated M13 Rev Primer. (A) PCR product after in vitro transcription reaction in duplicate reactions (lane 2 and 3). Two bands in lane 3 is due to the fact that products occasionally run as two bands after the in-vitro transcription; one at the expected size, and one that is double the expected size. (B) After annealing in duplicate reactions (lane 2 and 3). (C) Purified dsRNA of AsApoLp-III through Millipore column in duplicate reactions (lane 2 and 3).

In this way, dsRNA of ApoLp-III when injected inside mosquitoes trigger RNAi machinery and processed by RNAse III enzyme 'Dicer' to synthesize 21-24 bp small interfering RNAs (siRNAs). RNAi inducing silencing complex (RISC) binds these siRNAs. The guide strand of siRNAs helps RISC to target the corresponding mRNA. Argonaute protein (Argo) present in RISC complex cleaves the ApoLp-III mRNA. The cleaved target mRNA is amplified by RNA dependent RNA polymerase (RdRp) to form dsRNAs, which enter in RNAi pathway and hence silence the gene.

4.2.4.1 Silencing of AsApoLp-III gene in *An. stephensi*

ApoLp-III gene was silenced to examine how it effects the development of *Plasmodium* in *An. stephensi* or other gene. The effect of silencing of AsApoLp-III was evaluated by systemic injection of dsRNA. As a control, female mosquitoes were injected with dsLacZ, a gene not present in mosquito genome. After injection, the two groups were housed identically to eliminate all possible confounding factors.

At 4 days after injections, 10 mosquitoes were collected for RNA isolation and cDNA preparation. Silencing of AsApoLp-III was checked, through real time PCR using S7 as an internal control. The primers (Fig. 4.1; shown in arrow lines) used for checking the silencing were designed from region exclusive of the cloned plasmid used to prepare ds-RNA to avoid false positives. The primer sequence (5'to 3') for checking silencing was as: AsApoIII-Sil-Fw: GCGAAGCTTCTGTGCCTTAT AsApoIII-Sil-Rev: CTCGAATGCCACCTCAATCT. The transcription levels of AsApoLp-III were reduced

in mosquitoes injected with dsAsApoLp-III, relative to those injected with dsLacZ. The expression level of AsApoLp-III mRNA were significantly reduced by 84% (*p value* < 0.005) when mosquitoes were injected with their respective dsRNA (Fig. 4.8).

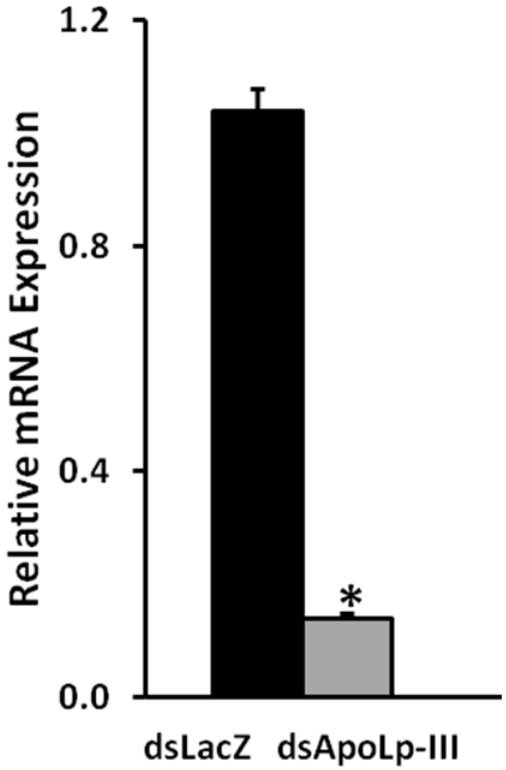

Fig. 4.8 Relative abundance of AsApoLp-III mRNA in *An. stephensi* mosquitoes injected with AsApoLp-III dsRNA and control LacZ dsRNA

In the complete *Plasmodium* life cycle (in both human and mosquito hosts), parasite numbers reaches its lowest during the oocyst stage which then rapidly increase when oocyst burst to release 1000's of sporozoites. For this reason, the midgut stages of parasite development constitute prime targets for strategies aiming to block malaria transmission. There is evidence that the mosquito can sense the presence of malaria parasites shortly after the ingestion of the blood meal (Vlachou et al., 2006, Dong et al., 2006), but it appears that the primary midgut immune response is triggered in response to ookinete invasion (Vlachou et al., 2006; Dong et al., 2006). Therefore, the entire study was carried out keeping these facts into consideration and to find out the role of ApoLp-III in *An. stephensi* particularly during this vulnerable period of *Plasmodium* life-cycle. Guided by the

transcriptional response of ApoLp-III during crucial hours of infection (Fig. 4.4A), it was certain that AsApoLp-III have a role to play in *Plasmodium* development, whether it be positive or negative. Therefore, we tested the effect of ApoIII on *Plasmodium* development. For that 1-2 days old female *An. stephensi* were injected with dsApo-LpIII and dsLacZ as control. 4 days later, both groups (dsLacZ and dsApoLp-III injected) were allowed to feed on *P. berghei* infected mice having 5-6% parasitemia and 1-2 exflagellation/field.

Midguts of these silenced mosquitoes were dissected 24 hours post infection to find out the silencing of ApoLp-III as well as status of immune response during ookinete invasion. This is the time when maximum induction of immune genes occurs as a part of response against parasite invasion. It was observed that expression of NOS showed drastic induction during the ookinete invasion in the midgut of AsApoLp-III silenced (*p value*<0.044) mosquitoes 24 hour post infection (Fig.4.9). In fact, NOS is a well-documented antiplasmodial molecule and the ookinetes must migrate out of the midgut cell before they are damaged by the action of this protein (Han and Barillas-Mury, 2002; Kumar et al., 2004; Gupta et al., 2005; Kumar et al., 2010). Thus, it might be possible that the early event of epithelial invasion by the ookinetes is also negatively affected in AsApoLp-III silenced midguts due to the toxic effect of NOS.

Further observation shows that AsApoLp-III silencing lead to decrease in the median number of live oocysts by 7 fold (*p value*<0.0001) 7 days post infection. Results presented in Fig. 4. 10 revealed that the variable number of oocysts were observed in controls (oocysts range 0-454) and silenced (oocysts range 0-580) midguts. However, the median value for the oocysts numbers in controls and AsApoLp-III silenced midguts was 109 and 33, respectively (Fig.4.10). The result clearly indicates that AsApoLp-III is required for effective infection and hence acts as *Plasmodium* agonist. We propose that AsApoLp-III might be transporting the lipids from hemolymph to the developing oocysts and in its absence this stage of parasite become more susceptible to the immune attack and subsequently undergo degeneration. These assumptions are supported by the previous study where developing *Plasmodium* oocysts are reported to be dependent on mosquito lipids (Atella et al., 2009).

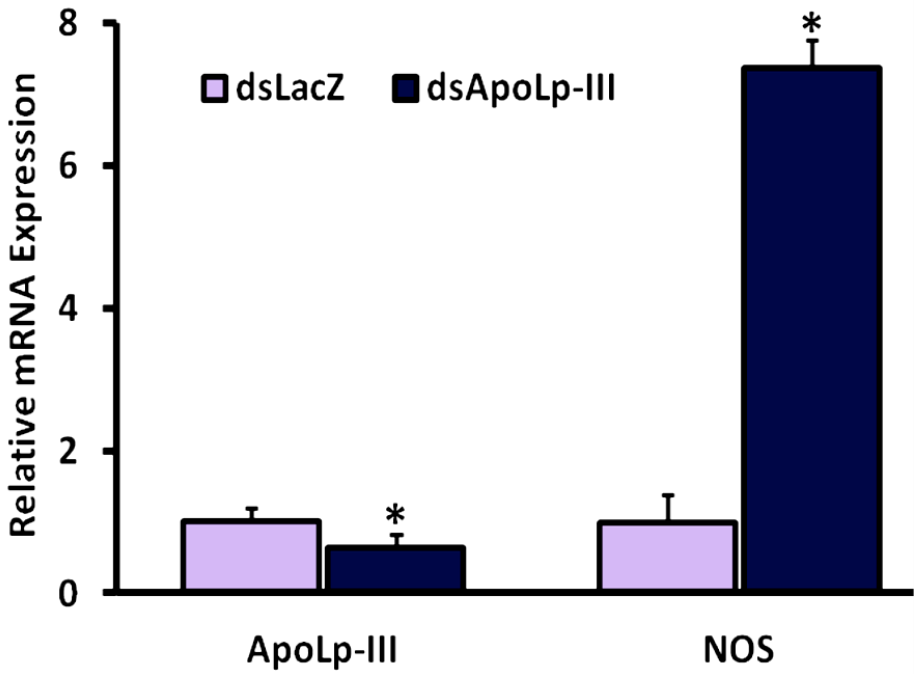

Fig. 4.9 Effect on NOS upon AsApoLp-III silencing 24 hour post *Plasmodium* feeding: First two bar shows the 36% decrease (*p value*<0.044) in ApoLp-III expression in midgut infected with *P. berghei* as compared to control. In the next two bars, NOS can be seen induced 7 folds in the midguts that have reduced ApoLp-III expression as compared to control.

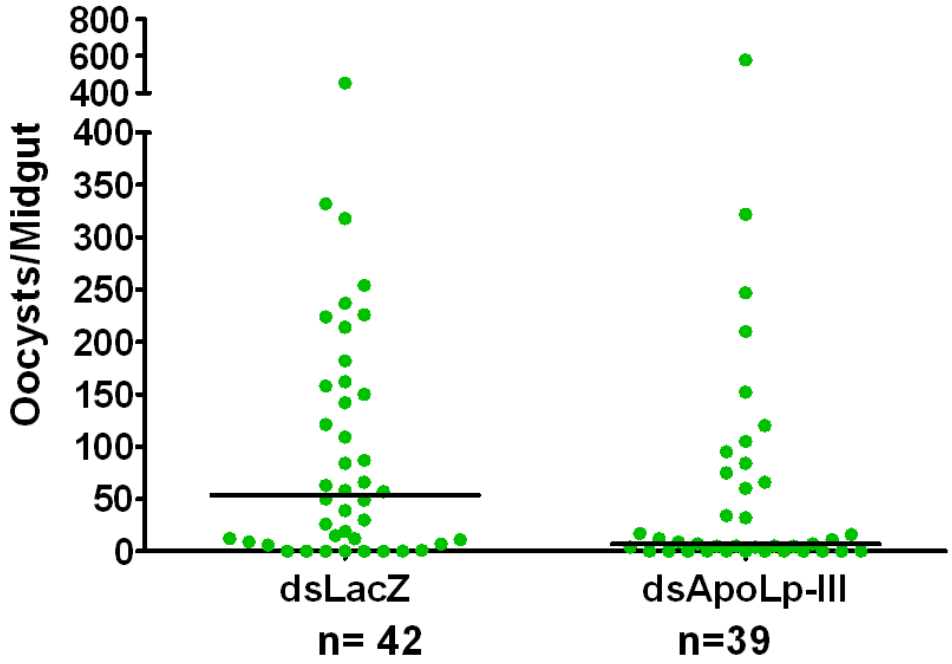

Fig. 4.10 Effect of AsApoLp-III silencing on the number of live oocysts (green dots) in midguts analyzed seven days post infection. Dots represent the number of parasites present on individual midguts, and the median number of parasites is indicated by the horizontal line. Distributions are compared using the Kolmogorov-Smirnov test; n = number of mosquitoes.

Advanced studies are essential to establish the mechanism through which ookinete migration and development rely on lipoproteins. A suggestive hypothesis can be made where ApoLp-III may escort to release ookinetes from the midgut epithelium as a secondary response of lipid transfer which is its primary function (Billingsley and Lehane, 1996). Thus, depletion of ApoLp-III might lock these ookinetes in the lethal milieu of the invaded midgut. This hypothesis is further supported by the strong induction of NOS in the reduced endogenous AsApoLp-III mRNA level in midgut at the time of ookinete invasion. This clearly proves that AsApoLp-III when presents shield the parasite against the immune attack and facilitates its easy transfer across the midgut epithelial.

4.3 Conclusion

India contributes 70% of malaria cases and 69% of malaria deaths in the South-East Asia region. *An. stephensi* is one of the major vectors responsible for malaria transmission in this region. Transmission blocking with the utilization of vector immunity is the most appealing approach as *Plasmodium* undergoes an important bottleneck situation during its development in the mosquito host. Molecular interactions between mosquitoes and malaria parasites studied in the laboratory models identified several mosquito immune-related genes, which affect the resulting infection in either positive or negative manner.

Here, we have discussed the positive regulatory effect of Apolipophorin-III (ApoLp-III) gene in *An. stephensi* that plays a decisive role in the survival of *P. berghei*. Reduced endogenous expression of AsApoLp-III leads to the decreased development of oocysts. Thus, we propose that AsApoLp-III protein might facilitate an easy egress of the parasite through midgut epithelium and/or further development into the oocysts. This view is further strengthened with the increased level of NOS 24 h post infection in the ApoLp-III silenced mosquitoes. It can be conclusively said that in the absence of ApoLp-III *Plasmodium* are trapped in the toxic environment of *An. stephensi* midgut, which eventually leads to lower infectivity.

However, the decreased production of oocysts 7 days post infection in the midgut of *An. stephensi* is in contrast to the results obtained on silencing of AgApoLp-III in susceptible G3 and refractory L3-5 mosquitoes that showed increased number of ookinetes (Gupta et al, 2010) whereas silencing of ApoLp-III in *An. gambiae* Yaounde´ strain females showed no effect on parasite development (Mendes et al, 2008). This contrasting behavior of the

gene in different species as well as different strains of same species put in forth the view that there occurs a huge assortment of compatibility, defined by the degree to which the immune system restricts the infection between particular strains of mosquitoes and specific parasites strains.

The real mechanism by which ApoLp-III functions in *An. stephensi* is yet to be explored. Experiments conducted in this manuscript indicated that this gene is playing an influential role in *Plasmodium* development. Categorically, this is the first report of involvement of ApoLp-III gene in Indian susceptible strain *An. stephensi,* which is responsible for major malarial transmission in the country.

Chapter 5

Expression analysis of STAT pathway genes in Anopheles culicifacies

In this chapter, we characterized STAT pathway genes: the transcription factor STAT and the regulatory proteins SOCS and PIAS from major Indian malaria vector *Anopheles culicifacies*. The expression profile in different mosquito development stages as well as in midgut of female mosquitoes upon *P. berghei* infection has also been studied.

Molecular characterization of SOCS gene and its expression analysis on *Plasmodium berghei* infection in *Anopheles culicifacies*

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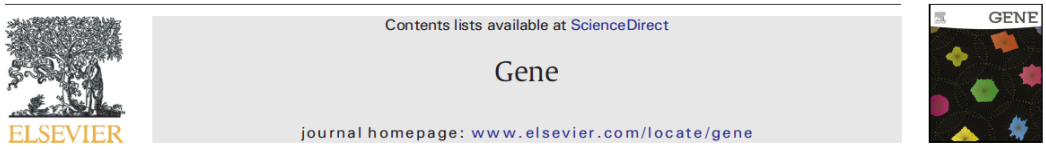
Gene 596 (2017) 89-97



Research paper

The evolutionary divergence of STAT transcription factor in different *Anopheles* species

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- Gupta K, Dhawan R, Kajla M, Misra T, Kumar S, Gupta L. The evolutionary divergence of STAT transcription factor in different *Anopheles* species. **Gene.** 2016, 596:89-97.

Chapter 5

5.1 Introduction

The *Anopheles* mosquito is known universally as the Malaria mosquito species. It is considered the primary vector of the *Plasmodium*, the causative agent of malaria. Despite this, it is well known that the mosquito mount vigorous innate cellular and humoral immune responses against *Plasmodium* parasite development. These immune responses are mediated through different signaling pathways. The major immune signaling pathways in mosquitoes are Toll, Imd and JAK/STAT (Waterhouse et al., 2007). However, the immune response generated for a particular pathogen varies from one species to another. For instance, we have seen in previous chapter that Apolipophorin in *An. stephensi* demonstrates an entirely different role in *Plasmodium* development than that of *An. gambaie* by acting as an agonist. This contrasting behavior of the gene in different species as well as different strains of same species indicate to the view that there occurs a huge assortment of compatibility, defined by the degree to which the immune system confines the infection between particular strains of mosquitoes and specific parasites strains. This brings in forth the need to understand the immune system of different mosquito species individually. Therefore, we are interested in understanding the JAK STAT immune signaling pathway in *An. culicifacies* which is responsible for more than 65% of malaria cases reported annually spread through this vector. It would be interesting to study the molecular mechanism of its role against *Plasmodium* development as the immune status of this species at molecular level is least explored.

In *Drosophila,* however, the JAK-STAT pathway is seen to be involved in several cellular processes such as regeneration, homeostasis, eye development and embryonic segmentation. Also, in *Drosophila* this pathway contributes in some cellular immune responses as differentiation of prohemocytes and hemocyte proliferation, along with antibacterial responses (Hanratty and Dearolf, 1993; Agaisse and Perrimon, 2004; Krzemien´ et al., 2007; Jiang et al., 2009).

Recent studies showed that the JAK-STAT pathway mediates *An. gambiae* immune response to *P. berghei and P. falciparum* (Gupta et al., 2009) and *Ae. aegypti* response to dengue virus II (Souza-Neto et al, 2009). Most of the components in JAK-STAT are conserved among dipteran insects. Given the multitude of roles played by the pathways, it is obvious to have multiple regulatory mechanisms that control them. In *D. melanogaster,*

STAT pathway is initiated when the peptide ligand Unpaired (Upd) binds to the transmembrane receptor Domeless. This activates the JAK kinase Hopscotch to phosphorylate the transcription factor Stat92E. The phosphorylated STAT protein forms a dimer which translocates into the nucleus and eventually activates transcription of target genes (Agaisse and Perrimon, 2004). This pathway is tightly regulated by negative regulators, such as Suppressor of Cytokine Signaling (SOCS) and Protein Inhibitor of Activated STAT (PIAS). The SOCS gene is transcriptionally activated by the STAT pathway as part of a negative feedback loop that modulates STAT signaling by preventing STAT phosphorylation, while PIAS inhibits signaling by directly binding to STAT proteins and targeting them for degradation (Wormald and Hilton, 2004) (Fig. 5.1).

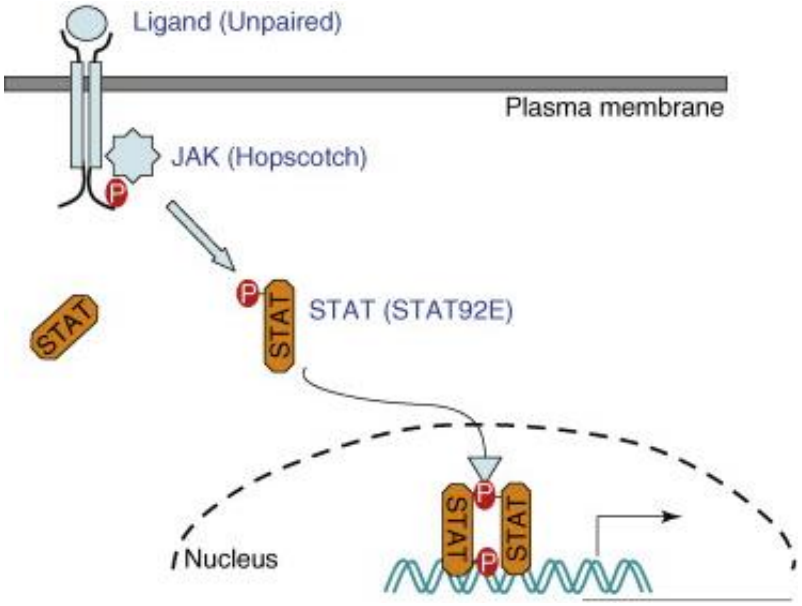

Fig 5.1 Mechanisms of JAK-STAT Signaling Pathway in *Drosophila*: Binding of ligand to its receptor Dome induces JAK-mediated STAT tyrosine phosphorylation and dimerization. STAT dimers translocate to the nucleus and bind to responsive DNA elements in the promoters of target genes and initiate gene expression (Ref: Li Willis X, 2008).

5.1.1 Role of STAT signaling pathway in development of insects

D. melanogaster has been extensively used as a representative organism in developmental research. These studies have revealed the essential role of the JAK-STAT pathway in many developmental processes (Harrison et al., 1995). The first evidence that the JAK-STAT pathway involved in development came from the characterization of dominant gain-of-function alleles of hop (Perrimon and Mahowald, 1986; Williams, 2000). The massive

amount of roles played by this signal transduction pathway regulated through multiple regulatory mechanisms. Its role starts with sex determination and segmentation and subsequently the formation of the tracheal pits, elongation of intestinal tracks and formation of the posterior spiracles in *Drosophila* (Arbouzova and Zeidler, 2006). Other, less well defined roles include: development of axons within the central nervous system and embryonic dorsal/ventral patterning (Ihle et al., 1996). This pathway is also needed for eye development, cell proliferation, photoreceptor differentiation and establishment of equator and ommatidia polarity (Luo et al., 1999). Classical embryological studies in *Drosophila* revealed broad developmental similarities exist among diverse dipteran species (Goltsev et al., 2007). In this chapter, the information about conserved roles of the STAT pathway during mosquito development in view of their similar and unwavering genomic array of developmental gene is considered.

5.1.2 Role of STAT signaling pathway in insect immunity

Insects have an evolutionarily conserved first-line host defence that comprises the senses of pathogenic microorganisms through pattern-recognition receptor (PRR) molecules (Hoffmann and Reichhart, 2002; Shuai and Liu, 2003). Innate immune system not only combats foreign invaders but it is also employed in wound healing, stress responses and the management of microbial symbiotic populations (Loker et al., 2004). However, memory-like responses have been reported in some insects that are termed as "immune priming" (Pham et al., 2007). There are examples of nonspecific and pathogen-specific priming in *An. gambiae* that can be long-lasting. Very recently a molecular mechanism has been elucidated, which allows the innate immune system to 'remember' a previous encounter with a pathogen (Ramirez et al., 2014). Challenged mosquitoes constitutively discharge a soluble haemocyte differentiation factor (Lipoxin/Lipocalin complex) into the hemolymph, which when transferred into naive mosquitoes, ultimately induces priming (Rodrigues et al., 2010; Ramirez et al., 2015). The signaling pathway(s) that mediate the establishment of immune priming are not known.

In *Drosophila*, STAT92E translocate into the nucleus of fat body cells upon bacterial challenge, which resulted into expression of several anti-microbial peptides including Tep and Tot protein families. Tep1 is one of the four members of the Tep family that contain thioester motifs and has high similarity to C3/α2-macroglobulin super family of human.

Tep1 expression was found highly induced in fat body cells upon immune challenge in *Drosophila* and *An. gambiae* (Lagueux et al., 2000; Levashina et al., 2001). Tep1 is also induced upon *Plasmodium* infection and responsible for lysis of ookinetes during evasion of mosquito midgut (Blandin et al., 2004; Blandin et al., 2008).

5.1.3 Immune Reports of STAT Pathway genes in Anophelines

Mosquitoes primarily rely on Toll, Imd, JAK-STAT and recently known JNK signaling pathways for limiting pathogen infection (Ramphul et al., 2015). Although Toll and Imd signaling pathways have been well studied against bacterial, fungal and *Plasmodium* infections (Dimopoulos et al., 2002; Meister et al., 2005; Blumberg et al., 2013), many evidences have recently been cited to describe the role of JAK-STAT pathway in anti-bacterial and anti-viral infections (Gupta et al., 2009; Souza-Neto et al., 2009). The first evidence that the JAK-STAT pathway also plays a role in insect immunity was shown in the mosquito *An. gambiae* where AgSTAT translocates into the nucleus of fat body cells in response to bacterial infection (Barillas-Mury et al., 1999). Moreover, *An. gambiae* STAT pathway display the late phase immunity against *P. falciparum* and *P. berghei* while Brazilian species, *An. aquasalis* show the early immune response against *P. vivax* infection (Gupta et al., 2009, Bahia et al., 2011). Studies carried in *An. aquasalis* mosquitoes indicate the induction of NOS is also paralleled with PIAS in midgut during *P. vivax*'s ookinete invasion (Bahia et al., 2011). Studies carried in *An. gambiae* also revealed similar findings that both NOS and SOCS5 are induced during *P. falciparum* and *P. berghei* invasion of mosquito midgut (Gupta et al., 2009). Although those two studies used different mosquito species and *Plasmodium* combinations however, these observations clearly indicate the involvement of STAT pathway in anti-plasmodial immunity and PIAS and SOCS5 being suppressants of this pathway is also induced during this process. Induction of mRNA expression of PIAS indicates STAT pathways is ON during ookinete invasion and in parallel the suppressor of this pathway is also active to counter balance the immune reactions. Moreover, in another *Anopheles* species, the induction of NOS and SOCS5 is also reported during midgut invasion by diverse *Plasmodium* species (Noh et al., 2006). Interestingly, the silencing of SOCS causes hyper activation of NOS which mediates *Plasmodium* killing in mosquito midgut (Gupta et al., 2009; Sharma et al., 2010; Vijay et al., 2011).

STAT pathway in *An. stephensi* has also been characterized in previous studies from our lab (Gupta et al., 2016). Quantitative PCR analysis revealed that AsSTAT-A and AsSTAT-B transcription factors mRNA levels are not affected after *P. berghei* infection. As expected, their expression is quite low and their requirement is just to switch-ON the pathways. They are expressing constantly in both control and infected samples but difference in their mRNA level is non-significant. In case of AsSOCS, near about 6 times higher expression was observed in the infected midgut whereas expression of AsPIAS was found significantly high in midgut compare to blood-fed control. This indicates that STAT pathway might be induced during ookinete invasion along with the suppressors of this pathway to fine tune the immune reactions. These findings are in agreement with other reports where STAT pathway genes and its suppressors are induced simultaneously at 24h after *Plasmodium* infection (Bahia et al., 2011; Gupta et al., 2009).

STAT signaling in Indian *Anopheles* species against *Plasmodium* infection has not been characterized yet. Literature supports that there exists much genetic diversity in *Anopheles* mosquitoes due to different geography and host-parasite interaction (Neafsey et al., 2015). Our study also revealed that STAT gene varies in different *Anopheles* species suggesting that it might act differently from one species to another species (Gupta et al., 2016). Immune genes, in general evolve at faster rate than whole genome, which is explained by the persistent selective pressures posed by new type of pathogens (Obbard et al., 2009). Therefore, in this chapter, we characterized genes from the JAK-STAT pathway: the transcription factor STAT and the regulatory proteins SOCS and PIAS in *An. culicifacies*. The expression profile in different mosquito development stages as well as in midgut which is the prime immune organ of female mosquitoes upon *P. berghei* infection has also been studied. It is to be noticed that *An. culicifacies* mosquitoes transmits both *falciparum* and *vivax* malaria in India.

5.2 Result and Discussion

5.2.1 Identification and Cloning of STAT pathway genes

Initially due to unavailability of genome sequence of *An. culicifacies* (before 2013) degenerate primers were designed (Table 5.1) on the basis of conserved regions of STAT, SOCS and PIAS with the help of known amino acid sequences of *An. gambiae*, *An.*

aquasalis, *Ae. aegypti*, *Ap. melifera* and *D. melanogaster*. Only insect species have been taken into the consideration to ensure accurate and less degeneracy in primers.

Table 5.1 List of degenerate primers of STAT pathway

S.No.	Deg Primers	Primer Sequence (5'→3')
1a	DegSTAT-A-Fwd	GARAADCARCCRCCRCARGTSATG
1b.	DegSTAT-A-Rev	GTRAAYGGCTGRATGTGYAGDAYCTG
2a.	DegSOCS-Fwd	GCCGCTCGAGCCIGHIGGIACITTYYTIGTIMGNGA
2b.	DegSOCS-Rev	GCCGCTCGAGTTYTGYTTRTARTGRTAYTCYTT
3a.	DegPIAS-Fwd	GCCGCTCGAGCARGTICARYTIAGRTTYTGYYT
3b.	DegPIAS-Rev	GCCGCTCGAGCCAIGTIGGYTTYYTYTCRTTCATYTG

For cloning of the STAT, SOCS and PIAS genes, gDNA or midgut cDNA were used as template. The PCR cycles were used as follows: two cycles (1 min steps at 95^0C, 55^0C and 72^0C, and 95^0C, 42^0C and 72^0C) followed by 40 cycles at moderate stringency (1 min steps at 95^0C, 52^0C and 72^0C) and a final 10 min extension at 72^0C. We followed the same PCR protocol for all degenerate amplification as per the earlier reports (Barillas-Mury et al., 1999). All amplicons generated were purified through Qiaquick PCR purification kit (Qiagen Cat No. 28104).

5.2.1.1 Identification, cloning and sequencing of SOCS gene of *An. culicifacies*

Suppressors of cytokine signaling (SOCS) protein function as negative regulator of STAT pathway and are involved in fine tuning of the immune responses. In contrast to other STAT pathway components, which exist as single members in invertebrates, three SOCS family members have been identified in fruit fly (SOCS36E, SOCS44A, SOCS16D) (Karsten et al., 2002). Among these three; SOCS36E is the orthologous to human SOCS5 and functions as negative regulator in JAK-STAT signaling pathway. SOCS protein, in general, contains three distinct regions, highly conserved SH2 domain, C-terminal SOCS box and N-terminal region. Each domain performs specific function and ultimately creates an inhibitory feedback loop for the JAK-STAT pathway (Babon et al., 2009; Stec et al., 2013).

Cloning of AcSOCS gene was done through degenerate primers designed from most conserved domains containing SH2 and SOCS box from *An. culicifacies* gDNA. These primers were designed to amplify sequences corresponding to two terminal stretches of 7-9 amino acids residues, PEGTFLLRD and KEYHY(K/R)Q, which are highly conserved

among many insect SOCS genes and amplified 436 bp fragment using gDNA (Fig 5.2 A and B). The cloned fragment was sequenced and its nucleotide identity was confirmed through BLAST with available gene sequences in NCBI. The cloned SOCS fragment (436 bp) contains partial SH2 domain and full length SOCS box domain. The gDNA sequence of AcSOCS was submitted to GenBank with accession number KJ914628. The sequence thus obtained was used to design gene specific primers (AsSOCS) that were used for q-PCR analysis. These were AcSOCSgspFwd: 5'-CGTCGTACGTCGTATTGCTC-3'and AcSOCSgsp Rev: 5'-CGGAAGTACAATCGGTCGTT-3' which yielded an amplicon size of 240 bp with cDNA and 315 bp with gDNA revealing the presence of intron between the two primers (Fig. 5.3A).

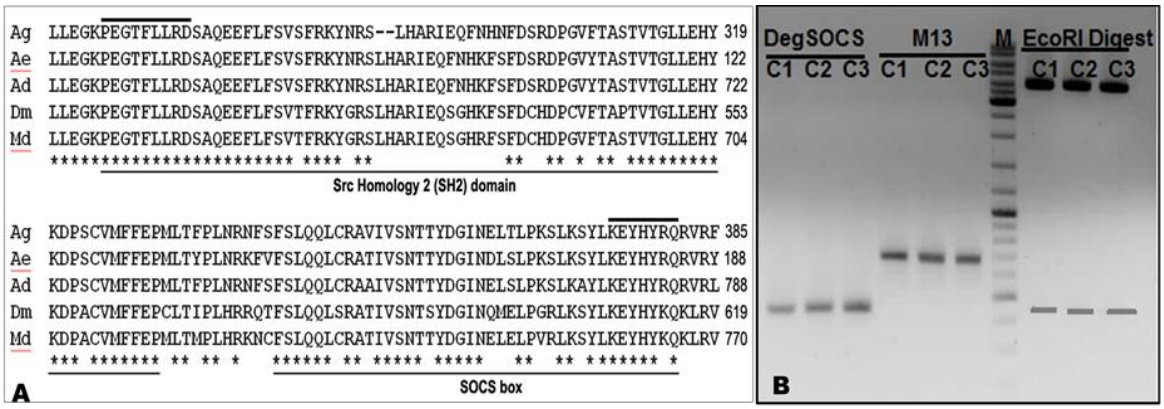

Fig. 5.2(A) Protein sequence alignment of SOCS protein of different insect species includes Ag, *Anopheles gambiae* (ABV01933.1); Ae, *Aedes aegypti* (XP_001656067.1); Ad, *Anopheles darlingi* (AND_003361); Dm, *Drosophila melanogaster* (AAO39436.1); Md, *Musca domestica* (XP_005179585.1). Thin underline in bottom represent the boundaries of SH2 and SOCS box domains. Thick line on the top reveals conserved amino acids sequences where forward and reverse degenerate (DegSOCS) primers were designed. (B) Verification of AcSOCS5 clones through degenerate SOCS (DegSOCS) primers (lanes 1, 2 and 3; expected size 436bp) and with M13 primers (expected size 656bp) using three bacterial colonies (C1, C2 and C3) as templates. Last three lanes are showing the EcoRI digestion of plasmids isolated by the same colony. 1 Kb ladder was used as a reference.

During that time, unannotated genome assembly of various *Anopheles* mosquitoes including *An. culicifacies* (Taxid No. 139723; Anop culi species A-37 1 V1 GenBank assembly [GCA 000473375.1]) became available at NCBI database.

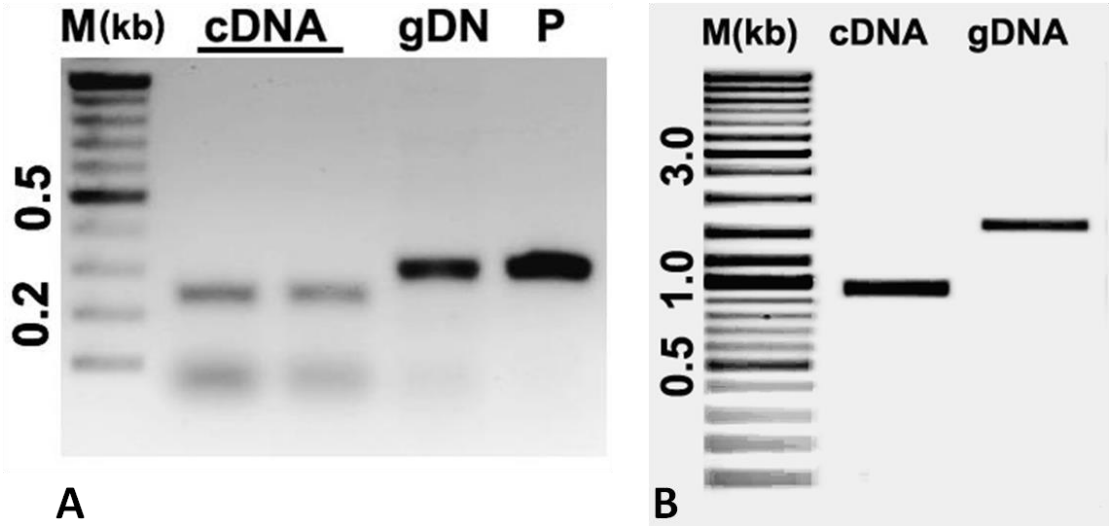

Fig.5.3 (A) Confirmation of intron in SH2 domain of *An. culicifacies* genomic DNA clone. *An. culicifacies* genomic or cDNA templates were amplified using gene specific (AcSOCSgsp) primers as shown in figure 1c. Lane 2 and 3 represent PCR amplified product from cDNA (expected size 241 bp) in duplicates and lane 4 from genomic DNA (gDNA, expected size 306 bp) template. In lane 5 the Ac-gSOCS cloned plasmid (P) template was used with same primers as positive control. In lane 1 DNA ladder was used as reference for identifying the size of amplified DNA fragments. (B) PCR amplified full length *An. culicifacies* genomic and cDNA using gene specific (AcSOCS-975bp) primers. *An. culicifacies* cDNA (lane 2, expected size 975 bp) or genomic (lane 3, expected size 1556 bp) templates were amplified. Lane 1 represents ladder with known molecular weight DNA fragments.

Sequence analysis of AcSOCS with *An. culicifacies* genome database confirmed the presence of three exons separated by two introns (Fig. 5.4A) of 516 bp in the N-terminal region and 65 bp in the SH2 domain (Fig. 5.3A).

Sequence analysis of deduced AcSOCS reveals the presence of SH2 and SOCS box domains and N-terminal region (Fig. 5.4B). The SH2 and SOCS box domains of AcSOCS showed 94–98% similarity with mosquitoes and 73–82% similarity with other insects; including *Drosophila* SOCS36E and human SOCS5 using BLASTP suit. It indicates that SH2 and SOCS box domains of AcSOCS are highly conserved during evolution due to their important role in receptor signaling.

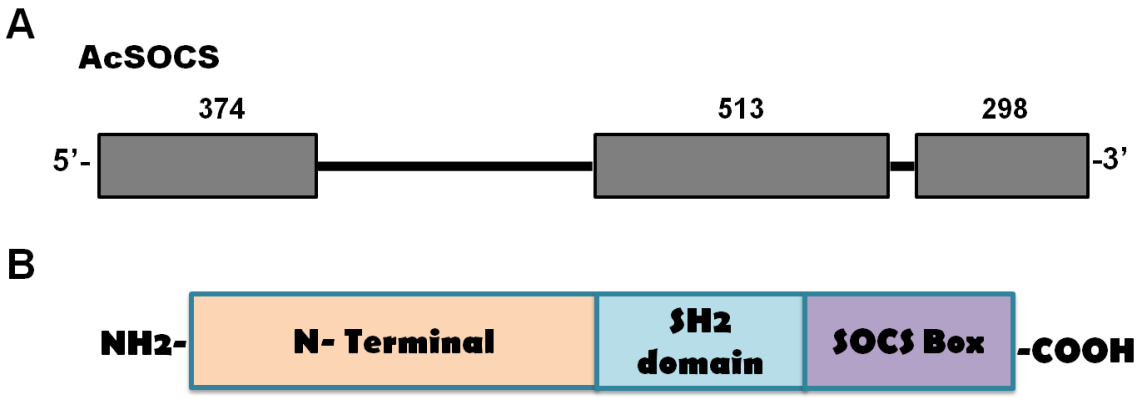

Fig.5.4 (A) Schematic organization of SOCS gene in *An. culicifacies* (5'→3'). (B) Conserved domains organization of SOCS gene.

Interestingly, the deduced amino acids sequence for AcSOCS N-terminal region (169aa) was checked for its similarity with other insect genome database using BLAST. It reveals poor identity with other insects and no relevant identity with *Drosophila*. However, it exhibits closest amino acids identity exclusively with Anopheline SOCS proteins. Further to compare AcSOCS N-terminal with other Anophelines sequences, different Anophelines N-terminal sequences were retrieved and curated manually to bring them in single reading frame. The deduced ORFs were then pairwise aligned to get % similarity (Table 5.2). These observations indicate that AcSOCS N-terminal amino acids identity is solely similar, rather limited to, Anopheline SOCSs. This variability may indicate that all the domains of AcSOCS experienced differential selection pressures and it provides the evidence that N-terminal domain is under least selection pressure (Wang et al., 2010). This is in agreement in other organisms such as *Drosophila*, where SH2 and SOCS box domains are conserved and N-terminal is most divergent (Stec et al., 2013).

Table 5.2 Percent identity and similarity of AcSOCS N-terminal domain. A sequence of 169 amino acids from N-terminal domain of AcSOCS was blasted and aligned with numerous SOCS protein from other organisms. Differential amino acids coverage for each of the sequence has been also presented here.

Mosquito Name	Accession No.	Amino Acid covered	% Identity	% Similarity
An. stephensi	ALPR02003551	169	86	90
An. dirus	APCL01002080	169	86	87
An. epiroticus	KB670658.1	169	80	83
An. farauti	KI915045.1	169	77	81
An. arabiensis	KB704895	169	77	80
An. darling	ADMH02001680	169	76	86

An. funestus	KB668345	169	75	79
An. merus	KI915156.1	169	75	79
An. gambiae	NT_078267.5	169	74	78
An. quadriannulatus	KB667888.1	169	74	78
An. christyi	KB703082	169	73	77
An. melas	AXCO02006542	169	73	77
An. atroparvus	KI421893.1	169	68	71
An. albimanus	KB672424.1	169	61	69
An. sinensis	ATLV01006259	169	58	61
Culex quinquefasciatus	AAWU01004291.1	69	55	68
Aedes aegypti	AAGE02000605.1	95	46	54
Lutzomyia longipalpis	AJWK01032776.1	99	41	48
Phelobotumus papatasi	AJVK01087732.1	107	38	45

Putative AcSOCS gene was used to design primers as AcSOCS-975bp Fwd: 5'-GACCATCATCCGTCGTTCTT-3'and AcSOCS-975bp Rev: 5'-CCGCTGCCGGTAGTGATACTC-3'. They were used to clone 975 bp SOCS fragment using cDNA as a template and sequenced it (Fig. 5.3B). Its sequence identity was confirmed through BLAST and thereafter submitted to GenBank with an accession number KJ914629.

5.2.1.2 Identification cloning and sequencing of *An. culicifacies* PIAS gene

Transcriptional suppression plays a central role in regulation of eukaryotic gene expression. Protein factors that obstruct in DNA binding activity of a transcription factor by corepressors control the basal transcriptional machinery or remodeling chromatin (Horwitz et al., 1996). The PIAS proteins have been shown to function as E3 ligases that facilitate the SUMO modification of a number of transcription factors (Rogers et al, 2003; Shuai, 2006). There is a single PIAS gene present in both *Drosophila* and *Aedes*, whilst four PIAS members in mammals (PIAS1, PIAS3, PIASx, PIASy) (Betz et al., 2001). In other insects including *Anopheles*, the probability of having one PIAS gene is definite.

Based on sequence alignment of the highly conserved domain of PIAS proteins, degenerate oligonucleotides were designed and used to amplify *An. culicifacies* genes using cDNA as template (blood fed midgut). For PIAS, PINIT domain conserved sequence VEPKRPPRPVN and Zn finger motif NLVIDGYFQ conserved region were targeted (Fig.

5.5 A). We successfully amplify the 528bp AcPIAS fragment from cDNA template which was further purify and cloned into TOPO TA Vector (Fig. 5.5 B).

The sequencing result of these clones was shown the maximum identity with *A. gambiae* and *Ae. aegypti* and *An. aquasalis* of respective genes. The sequence obtained was utilized for designing of gene specific primers for real time analysis. The primers used for q-PCR analysis were: AcPIAS Fwd: 5'- ACAACGACGCATCAAAGCAC -3' and AcPIAS Rev: 5'- GTGTCCGTTGCCGAATCCTA -3'.

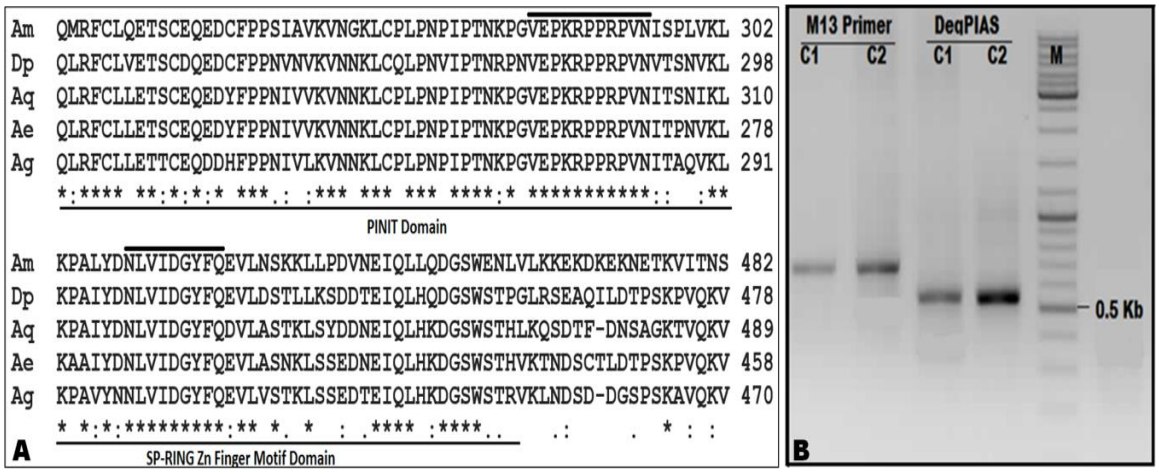

Figure 5.5 (A) Degenerate primers sequences of PIAS genes designed from conserved region of the protein. Protein sequences for different insects were obtained from NCBI database. The numbers on left side correspond to amino acid residue in particular protein. Sequence alignment of PIAS protein of different insect species include Am, *Apis mellifera* (XP_623571.3); Dp, *Drosophila pseudoobscura* (XP_002138569.1); Aq, *Anopheles aquasalis* (AEK26394.1); Ae, *Aedes aegypti* (XP_001647815.1); Ag, *Anopheles gambiae* (XP_001688469.1). Thin underline in bottom represents the boundaries of PINIT and SP-RING Zn finger motif domains. Thick line on top reveals conserved amino acids sequences where forward and reverse degenerate (DegPIAS) primers were designed. (B) Confirmation of *An. culicifacies* PIAS (AcPIAS) clones through M13 and DegPIAS primers. 1 Kb ladder (SM0331) was used as a reference for each gel image.

We followed the same strategy to retrieve the PIAS gene from the genome assembly of *An. culicifacies* mosquitoes. Here, *An. stephensi* PIAS was used as query to find out the probable contig that contain AcPIAS (AXCM01005731). BLAST-WGS search showed 95% nucleotide identity with query coverage of 82%. The full PIAS genes in *An. culicifacies* showed 10 exons and 9 introns (Fig. 5.6A). The size of exons in *An. culicifacies* in 5' to 3' direction are 42bp, 164bp, 610bp, 241bp, 106bp, 129bp, 164bp, 98bp, 181bp and 74bp respectively that yield CDS of 1809 bp encoding 602 amino acids. The PIAS protein family contains several highly conserved regions (Fig.5.6B). The most striking conserved domain of the PIAS family is a MIZ/SP-RING-finger-like zinc-binding domain

(RLD) and the SAP (scaffold attachment protein-A/B, acinus and PIAS) domain. The N-terminal SAP domain is evolutionarily conserved in proteins ranging from yeast to human in origin. They share the origin with other chromatin-binding proteins, such as scaffold attachment factor A and B (SAFA and B) (Aravind and Koonin, 2000). The SAP domain can recognize and bind to AT-rich DNA sequences present in scaffold-attachment regions/matrix-attachment regions (S/MAR) and provide a unique nuclear microenvironment for transcriptional regulation (Kipp et al., 2000). Next to the SAP domain, the Pro-Ile-Asn-Ile-Thr (PINIT) domain is attached, which represents a highly conserved region of PIAS proteins and involved in the nuclear retention of PIAS (Duval et al., 2003). The C-terminal RING (Really Interesting New Gene)-finger-like zinc-binding domain (RLD) in the end of the protein is the most conserved region and is needed for the SUMO (small ubiquitin-like modifier) E3 ligase function of PIAS proteins (Hochstrasser, 2001; Rytinki et al., 2009). Adjacent to the RING domain, a SUMO interacting motif is there, which mediates noncovalent interactions with SUMO proteins (Johnson, 2004; Sharrocks, 2006).

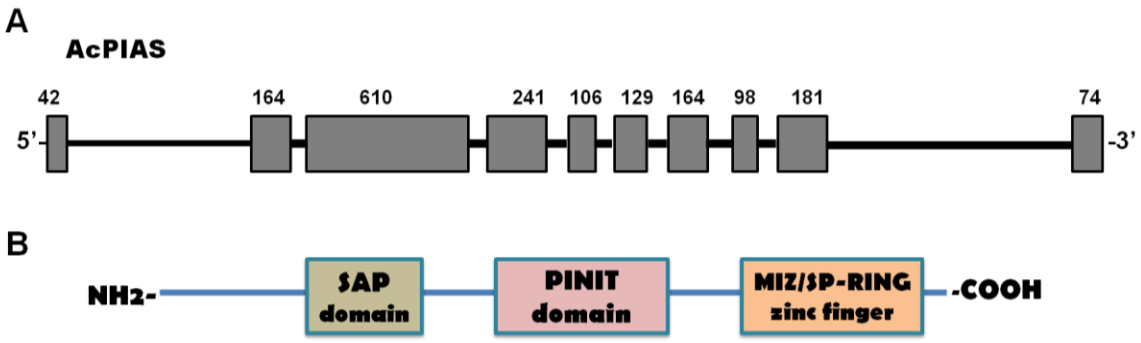

Fig.5.6 (A) Schematic presentations of genomic architecture of AcPIAS. (B) Conserved domains of PIAS gene.

5.2.1.3 Identification of *An. culicifacies* STAT-A gene

Currently, whole genome sequences of different *Anopheles* species (major and minor) from various geographical regions are available at NCBI. These whole genome shotgun (WGS) sequences can be used to sort out the genes present in the vast unannotated genome through simple bioinformatic tools (Neafsey et al., 2015). Here, *An. gambiae* STAT-A was used as query to find out the probable contig (AXCM01003811.1) that contain STAT-A gene in *An. culicifacies* (AcSTAT-A). BLAST-WGS showed 88% nucleotide identity with query coverage of 61%. BLAST search gave 7 matches that corresponds to 7 exon in AcSTAT-

A. This contig (AXCM01003811.1) was then subjected to GenScan to find out the exon-intron boundaries. After final verification, the predicted AcSTAT-A gene consisting of 2259 bp encoding 752 amino acids was submitted (NCBI Reference ID: BK009255). The seven exons of AcSTAT-A are of 128bp, 974bp, 124bp, 124bp, 490bp, 340bp and 79bp, respectively (Fig. 5.7A). The ORF generated matched with AgSTAT-A with an identity of 96%.

Conserved domains and motifs in these predicted proteins were determined using the programs, Pfam or Conserved Domain (CD) search. All *Anopheles* species have the same functional domain organization for STAT protein *viz.* STAT interaction domain, alpha domain, STAT binding domain and SH2 domain presented schematically in Fig. 5.7B.

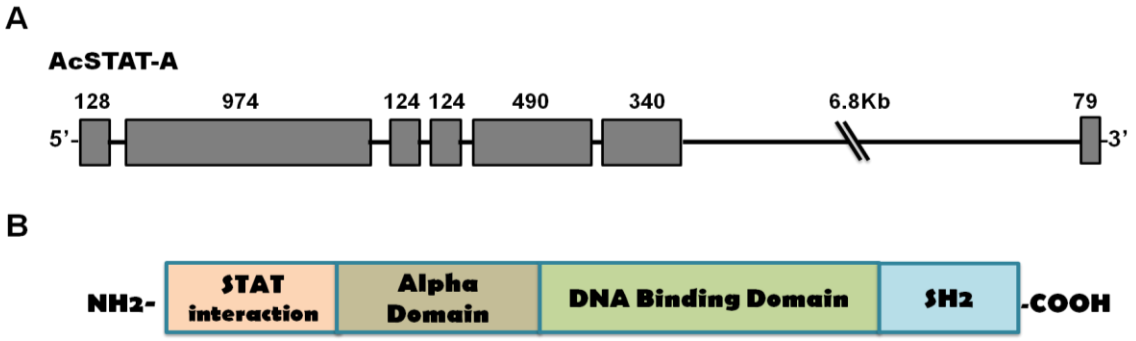

Fig. 5.7(A) Schematic presentations of genomic architecture of AcSTAT-A. (B) Conserved domains of STAT-A gene.

This predicted cDNA was used to design primers for q-PCR expression analysis. The primer set used for AcSTAT-A were: AcSTAT-Fwd: 5'-GCAACTTTCGCCAGCTATTC-3'and AcSTAT-Rev: 5'- CTTCATCAGGTTCCGGTTGT-3' which gave an amplicon size of 206 bp (Fig. 5.8).

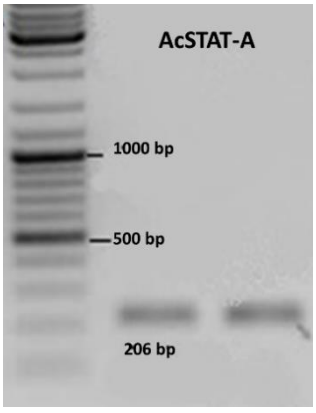

Fig. 5.8 PCR amplified *An. culicifacies* cDNA using gene specific (AcSTAT) primers (lane 2, 3 expected size 206 bp).

5.2.2 Expression of STAT pathway genes in different developmental stages of *An. culicifacies*.

It was well established fact that, STAT pathway participates in the developmental process of many insects including mosquitoes (Baumer et al., 2011; Bahia et al., 2011). Therefore, the involvement of STAT pathway in different developmental stages of *An. culicifacies* was investigated. Different developmental stages of mosquito life cycle *viz.* eggs, larvae, pupae and adult males and females were collected. Total RNA was isolated and their cDNA were prepared and these samples used for gene expression analysis. For expression analysis, set of primers for different STAT pathway genes are summarized below.

Table 5.3 List of Primers used for Quantitative PCR for different genes

S.No.	Primers	Primer Sequence (5'→3')	Amplicon size with cDNA
1a	AcSTAT-A-Fwd	GCAACTTTCGCCAGCTATTC	206 bp
1b.	AcSTAT-A-Rev	CTTCATCAGGTTCCGGTTGT	
2a.	AcSOCS-Fwd	CGTCGTACGTCGTATTGCTC	241 bp
2b.	AcSOCS-Rev	CGGAAGTACAATCGGTCGTT	
3a.	AcPIAS-Fwd	ACAACGACGCATCAAAGCAC	404 bp
3b.	AcPIAS-Rev	GTGTCCGTTGCCGAATCCTA	

Ribosomal protein subunit S7 (rpS7; housekeeping gene) was used as an internal loading control for normalization of the gene expression (Salazar et al., 1993). Semi quantitative real time PCR (qPCR) analysis indicated that STAT pathway gene expression was detected in all life stages of *An. culicifacies* (Fig. 5.9).

The up regulated STAT-A, SOCS and PIAS mRNA expression in eggs signify the activation of STAT pathway during embryonic development. This corresponds with the reports where STAT pathway is up regulated during insect embryonic development along with the expression of its own regulators. For example, in *Drosophila* SOCS36E mRNA is developmentally expressed, with relatively high expression in 2 and 12 hour embryonic stages. In addition, SOCS36E mRNA levels rapidly decline at the end of embryogenesis and return to basal level during larval stages of development (Mukherjee et al., 2006; Callus et al. 2002).

We also observed that during pupal stages of development, expression levels of STAT and SOCS are high compared to larvae (Fig. 5.9A and B). These findings correspond with other reports where STAT pathway is up regulated during insect metamorphosis and also induce

the expression of its own regulators. In Brazilian mosquito *An. aquasalis*, expression levels of both STAT and PIAS genes are higher in pupa compare to larval stages (Bahia et al., 2011).Mosquito pupa is in fact a transient stage of development where almost whole body reorganization takes place under the influence of hormones (Truman and Riddiford, 1999). This is a stage where cell death and regeneration occurs extensively. These findings also supported by others studies where the expression of STAT pathway and negative regulators are induced in mosquito pupae (Bahia et al., 2011; Callus et al., 2002). The induced expression of genes during *An. culicifacies* pupal development indicated that STAT pathway is also responsible for reorganization of the internal body parts during metamorphosis.

The expression of SOCS genes is comparatively higher in males than females that correlate this finding with other reports where SOCS is found to be a male biased gene in mosquito *An. gambiae* and *An. culicifacies* (Fig. 5.9B) (Magnusson et al., 2011; Dhawan et al., 2015). Similar expression is seen in STAT and PIAS genes of STAT pathway (Fig. 5.9A and C). These findings may support the concept that sex-regulated genes control gender dimorphism and determine the ability of male or female mosquitoes to perform specific behaviors such as, female precision to transmit malaria parasite. The study of such genes may certainly help in understanding the sexual development and also provides valuable targets for controlling fertility, altering the sex ratio and reducing mosquito population through genetic control measures.

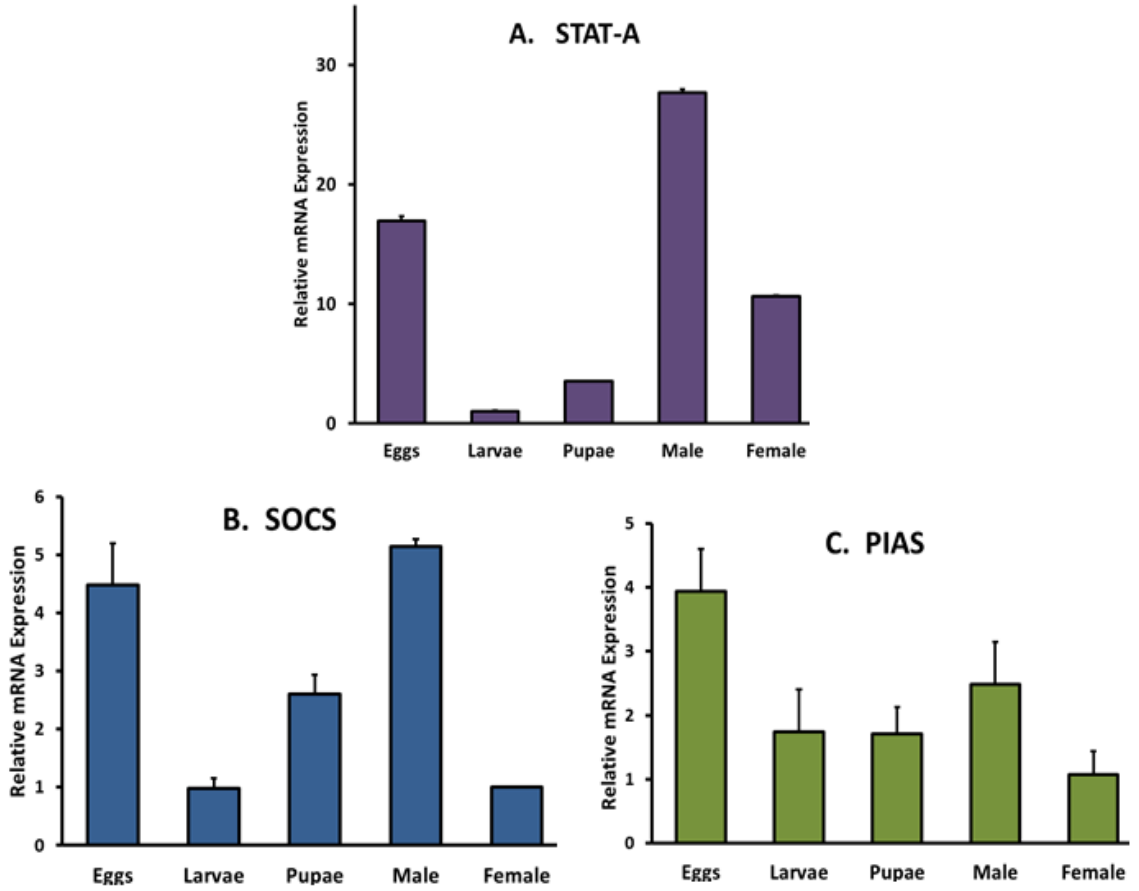

Fig. 5.9 Expression profile of STAT pathway genes was checked through real time analysis. The graph in the figure shows the expression of STAT pathway genes in different developmental stages of *An. culicifacies* with purple, blue and green color representing AcSTAT-A, AcSOCS and AcPIAS, respectively. Ribosomal S7 protein was amplified as reference.

5.2.3 Expression analysis of STAT pathway genes in response to *Plasmodium* in midgut of *An. culicifacies*.

The *Plasmodium* cycle commences when female *Anopheles* mosquito ingests gametocytes-infected blood. Ingested gametocytes differentiated into macro- and micro-gametes in the mosquito midgut immediately after blood feeding. These motile micro (male) gametes emerge from erythrocytes through a process called exflagellation (Billker et al., 1997). This is followed by the fertilization of macrogamete which leads to the formation of non-motile zygote. During 15-20 h post fertilization, the zygote differentiates into motile ookinete and penetrates the non-membranous peritrophic matrix. *Plasmodium* invades mosquito gut cells and encounters the immunity, which limits their growth and development (Baton and Ranford-Cartwright 2005). It may be a general phenomenon of interaction of *Plasmodium* with vector *Anopheles* species and defined as a strategy for parasite survival in host (Vinetz

et al., 2005; Vlachou et al., 2006). Among the variety of immune responses, STAT pathway plays a fundamental role by inducing the expression of NOS, which catalyzes the formation of nitric oxide (NO), a highly reactive immune molecule (Han et al., 2000). The NO gets converted to NO_2 which mediates protein nitration in a peroxidase-catalyzed reaction (Kumar et al., 2004; Oliveira et al., 2012). The alleviated production of NO not only bears out toxicity to the parasite, it can also be potentially deleterious to the host itself (Luckart et al., 1998; Han et al., 2000). Therefore, to ease the overall rigorousness of these toxic molecules, STAT pathway should be negatively regulated by PIAS and SOCS through a negative feedback loop.

These antiplasmodial roles of STAT pathway have been extensively studied in other insects and mosquitoes however, it is unknown in *An. culicifacies*. To understand the involvement of STAT pathway genes in anti-*Plasmodium* response, adult female mosquitoes were fed on a non-infected (control) or *P. berghei (Pb)*-infected mouse. After 24h of blood feeding (it is the time when *Plasmodium* ookinetes invade midgut epithelium) midgut were collected separately and the expression of STAT pathway genes were analyzed. The one-way ANOVA test was used as statistics method to calculate the significant difference. We investigated the effect of *P. berghei* (Pb) infection on expression of these three genes (STAT-A, SOCS and PIAS) in midgut (Fig. 5.10).

Quantitative PCR analysis revealed that AcSTAT-A transcription factors mRNA levels is not affected after *P. berghei* infection. As mentioned earlier that their expression is quite low and their requirement is to switch-on the pathways. They are expressing constantly in both control and infected samples but difference in their mRNA level is non-significant ($p\ value > 0.5$). In case of AcSOCS near about 3.5 times higher expression was observed in the infected midguts ($p\ value < 0.001$), whereas AcPIAS was found two times high in midgut ($p\ value < 0.01$) compare to blood-fed control (Fig. 5.10).

This indicates that STAT pathway might be induced during ookinete invasion and in parallel the suppressor of this pathway is also active to counter balance the immune reactions. These findings are in agreement with other reports where STAT pathway genes and its suppressors are induced simultaneously at 24h after *Plasmodium* infection (Bahia et al., 2011; Gupta et al., 2009).

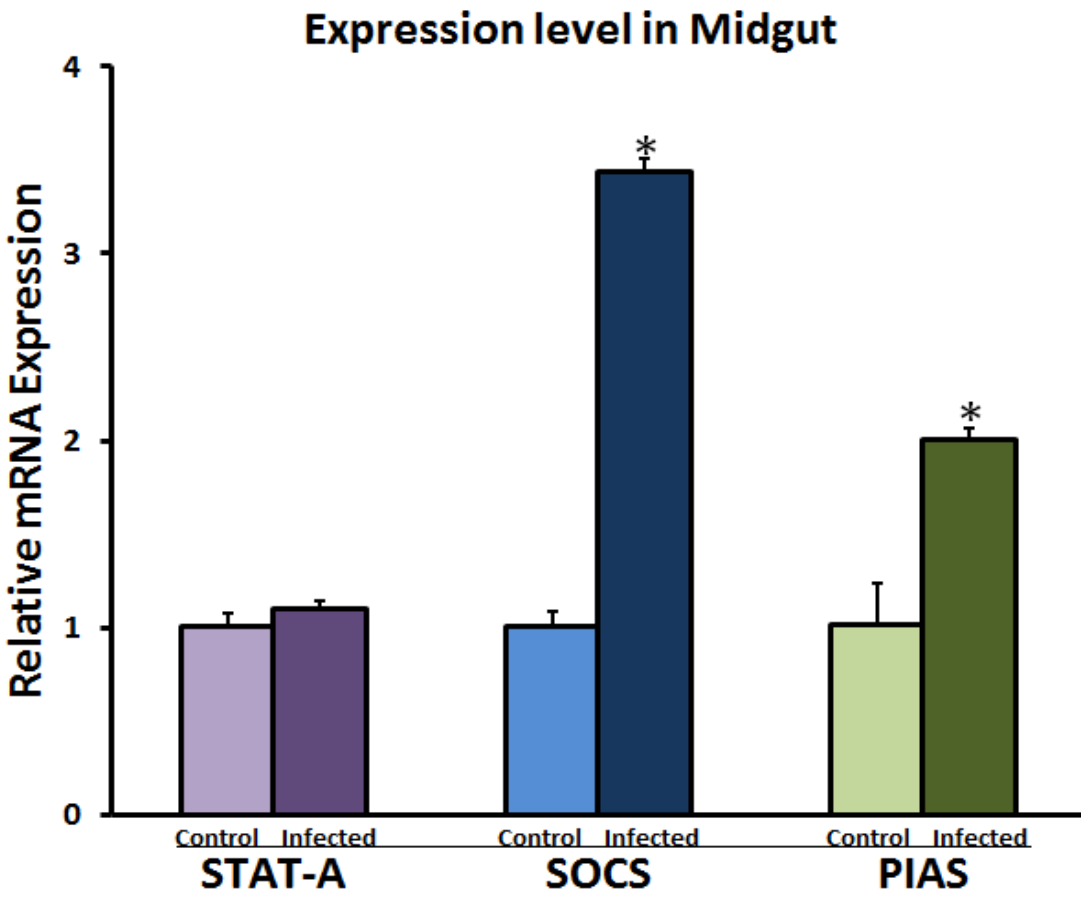

Fig. 5.10 STAT pathway gene mRNA expression in midguts of female mosquitoes fed on an uninfected and *P. berghei*-infected mouse. Purple, Blue and Green color represents AcSTAT-A, AcSOCS and AcPIAS.

Studies carried in *An. aquasalis* mosquitoes indicate the induction of NOS is also paralleled with PIAS in midgut during *P. vivax*'s ookinete invasion (Bahia et al., 2011). Studies carried in *An. gambiae* also revealed similar findings that both NOS and SOCS5 are induced during *P. falciparum* and *P. berghei* invasion of mosquito midgut (Gupta et al., 2009). Although those two studies used different mosquito species and *Plasmodium* combinations however, these observations clearly indicate the involvement of STAT pathway in antiplasmodial immunity. Also, PIAS and SOCS being suppressants of this pathway is also induced during this process. Induction of mRNA expression of PIAS indicates that STAT pathway is on during ookinete invasion and in parallel the suppressor of this pathway is also active to counter balance the immune reactions. Moreover, in another *Anopheles* species the induction of NOS and SOCS is also reported during midgut invasion by diverse *Plasmodium* species (Noh et al., 2006). Interestingly, the silencing of

SOCS causes hyper activation of NOS which mediates *Plasmodium* killing in mosquito midgut (Gupta et al., 2009; Sharma et al., 2010; Vijay et al., 2011). Dengue viruses (DENV-2) in *Aedes* mosquitoes exploit similar mechanism to evade host immunity (Souza-Neto et al., 2009). The midgut compartment of mosquito represents one of the most exigent environments for the survival and development of *Plasmodium*.

5.3 Conclusion

Among many known genes, STAT pathway genes are important as they have been involved in regulating developmental processes and immunity against a variety of pathogens (Agaisse and Perrimon, 2004). STAT pathway also regulated by the constitutive expression of SOCS and PIAS genes in a downstream process to negatively suppress the pathway. NOS play an important effector molecule of STAT pathway and subsequently exploited to regulate the transmission of vector borne pathogens (Gupta et al., 2009). In this chapter, we identified and characterize the components of evolutionary conserved STAT pathway genes from major Indian malaria vector *An. culicifacies*. We also analysed the expression of STAT pathway genes during developmental and *Plasmodium* infection.

Expression of STAT pathway gene throughout the developmental stages reveals its important role in development. Interestingly, the SOCS and other STAT pathway gene were found to be male biased gene in *An. culicifacies* and play an important role in controlling gender dimorphism and gender-based specific behaviours (Magnusson et al. 2011; Dhawan et al., 2015).

Expression analysis of each studied pathway components showing significant change after *Plasmodium* infection. The specific upregulation of effector gene in response to *Plasmodium* infection is suggestive of its role in contributing in immunity to the *An. culicifacies*. Our results clearly demonstrate the upregulation of SOCS, PIAS and NOS after *Plasmodium* infection in midgut of mosquitoes. The present study seems promising as it provides a worthwhile option for future studies as modulation of this pathway in the susceptible strain A of *An. culicifacies* which is one of the major malaria vectors in India and adjacent countries.

CONCLUSION

There are approximately 3,500 species of mosquitoes grouped into 41 genera. Human malaria is transmitted only by females of the genus *Anopheles*. Of around 450 *Anopheles* species, only 30-40 transmit malaria in nature. Some *Anopheles* species are poor vectors of malaria, as the parasites do not develop well (or at all) within them. Besides, there also occurs variation within species. Vector and parasite relationships are extremely specific and peculiar in nature which determines the dynamicity of parasite development and malaria transmission among humans (Sinka et al., 2012). It has been hypothesized that the molecular mechanism of a variety of malaria vector species varies with pathogen which might have different due to disproportional susceptibility (Molina-Cruz and Barillas-Mury, 2014). Recently, a landmark study was published by Molina-Cruz et al., 2015 in which they have proposed the lock-key theory, which implies that with different haplotypes of Pfs47 gene of *Plasmodium* strains, the malaria vector species of different geographical region behave differently. Why only certain species or strains of Anopheline mosquitoes are major malaria vectors and what determines the vector competence remains an enigma and warrants its elucidation. Therefore, this brings forth the importance to detail the immune responses generated in different vector species due to pathogen invasion. In the current work, immune responses of two major malaria causing vector species, i.e., *Anopheles stephensi* and *Anopheles culicifacies* have been studied at molecular level. It will help to enhance our knowledge about vector-parasite interaction in mosquito species of Indian sub-continent where malaria is still a major problem.

Some of the major conclusions from the thesis are highlighted as follows:

- Detailed *in-silico* prediction of ApoLp-III gene in *Anopheles stephensi* as well as identification of ApoLp-III gene in the genome of recently sequenced 16 *Anopheles* species was carried out. The putative ApoLp-III from these Anophelines were comparatively analyzed among themselves as well as with other insects and their phylogenetic relationship had also been studied. The phylogenetic analysis revealed the clear divergence of the gene from their lepidopteron and other dipterans counterparts.

- Through cloning and sequencing of Apolipophorin-III gene in *An. stephensi* its precise sequence and introns-exons boundaries were determined. Its role during *Plasmodium* infection within the mosquito had been analyzed in details. AsApoLp-III mRNA expression was strongly induced in *Plasmodium berghei* infected mosquito midguts at 3h and 24h, suggested its significant role in parasite development.

- Functional characterization of ApoLp-III in *An. stephensi* was accomplished using RNA interference technique. It was seen that AsApoLp-III silencing decreased *P. berghei* oocysts numbers by 7.7 fold against controls. These effects might be due to the interruption of AsApoLp-III mediated lipid delivery to the developing oocysts. Earlier studies of different strains of *An. gambiae* (Yaounde' and G3) with *P. berghei* showed either no effect or opposite effect (Mendes et al., 2008 and Gupta et, al., 2009) suggesting each *Anopheles* species may respond differently with same parasite.

- Nitric oxide synthase (NOS), an antiplasmodial gene, was also highly induced in AsApoLp-III silenced midguts suggested that AsApoLp-III acts like an agonist which aids in the development of *Plasmodium* and protects it against the mosquito immunity.

- STAT pathway genes: the transcription factor STAT and the regulatory proteins SOCS and PIAS were characterized in another major Indian malaria vector *Anopheles culicifacies*.

- The STAT pathway genes were present in all developmental stages of mosquito with prominence at initial egg stages. Also, the q-PCR analysis hints towards the male biasness of these genes.

- The effect on expression of STAT pathway genes in midguts of *An. culicifacies* mosquito 24 hour after *P. berghei* infection was investigated. Quantitative PCR analysis revealed that AcSTAT-A mRNA levels were

remain unchanged during *P. berghei* infection, however, AcSOCS and AcPIAS, higher expression was observed in the infected midguts compared to blood-fed controls. This indicated that STAT pathway might be induced during ookinete invasion and in parallel the suppressors of this pathway were also activated to counter balance the immune reactions.

- Cloning and Characterization of the genes encoding ROS detoxifying enzymes (Catalase and SOD) were analyzed in *An. culicifacies* mosquitoes. Their mRNA expression in all developmental stages of mosquito suggested that both genes were required in all stages.

- The reduced expression of CuZnSOD in *An. culicifacies* at 24h post *P. berghei* infection suggested that upsurge intracellular levels of free radicals in the carcass (rest of the body except midgut) may help in the clearance of *Plasmodium* load.

- AcCatalase mRNA levels were induced in the infected midgut and carcass as compared to control. This might be the outcome of two prospects; either the mosquito tends to lower H_2O_2 generated as an immune response to normal levels in their body, a probable step to neutralize increased production by the activated immune system, or it might be a strategy of *Plasmodium* to modulate the immune response of mosquito.

FUTURE PROSPECTIVE

The scope of this thesis was to identify the important immune genes which were playing a crucial role in mosquito immune system against *Plasmodium* infection. The focus of the thesis was to decipher the immune response of different mosquito species against *Plasmodium* infection. A further research will include the functional elucidation of following future perspectives:

- ApoLp-III comprises of alpha helices arranged in up and down topology. It would be interesting to know the mode of action of this protein in mosquito hemolymph during pathogen interaction.

- It would be interesting to know the effect of silencing of AsApoLp-III on other immune genes to understand the molecular mechanism that aids in development of *Plasmodium* infection.

- ApoLp-III is a multi-functional protein and is found to be playing crucial roles in other insect species. The interaction of ApoLp-III with ApoLp-I/II in transfer of lipid during flight and also finding out the role of this gene in fecundity of mosquito is a subject of greater interest.

- After the establishment of their role in *Plasmodium* development, functional characterization of STAT pathway gene in *An. culicifacies* can be the future refinement of this thesis.

- Characterization and Expression analysis of ROS detoxifying enzymes genes in *An. culicifacies* against *Plasmodium* had been discussed. It would be interesting to functionally analyze these detoxifying genes during infection through RNAi.

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BRIEF BIO-DATA OF SUPERVISOR

Prof. Lalita Gupta received her Ph.D. degree in Molecular Biology from Banaras Hindu University in 1999. She has extensive research experience of diverse research area of contemporary Biological Science world. She started her post-doc career at Colorado State University, Fort Collins, Colorado, USA and later moved to NIAID, NIH, USA as a research scientist with Prof. Carolina Barillas-Mury. She was working on molecular interaction of mosquitoes with *Plasmodium* or Dengue virus infection and investigating the effect of mosquito immune system on malaria disease transmission.

During this period she earned several research papers of international reputation including Science, PNAS and Cell host and Microbes. She also received several awards including best performance award from NIH. She joined BITS Pilani in 2008 as an Assistant Professor in Biological Sciences group and was promoted to Associate Professor in Department of Biological Sciences in 2013. In BITS Pilani, she was involved in interactive teaching of first degree (M.Sc. Hons.), higher degree (M. E. Biotech) and Master of Public Health courses. She acquired the designation of convenor of Departmental Research Committee and senate member of institutional research board and proctorial committee. She has so far supervised two PhD student and several higher degree students for their research projects. Prof. Gupta established a lab in the Biological Department with huge amount of funds from various national funding agencies. She has successfully completed two of her research projects from ABG and DST-SERB as a principal investigator. Currently she has major grants from ICMR, DBT and DST.

She has been actively participating in several esteemed National and International Conferences. She was awarded with DST Travel grant, 2012 for attending International Congress of Entomology, 2012 in South Korea where she was rewarded with "Outstanding achievement award". Besides, she has continually presented her work in internationally reputed Conferences like American Society of Tropical Medicine and Hygiene (ASTMH) and Gordon Research Conference.

Recently, she joined as Professor and Head of Department of Zoology in Ch. BansiLal University, Bhiwani, Haryana (September, 2016). She is also adorning the post of Dean, Life Sciences. She is actively working in the upliftment of research in this recently established Government Institute.

Distinguished Publications:

Gupta L, Noh JY, Jo YH, Oh SH, Kumar S, Noh MY, Lee YS, Cha SJ, Seo SJ, Kim I, Han YS, Barillas-Mury C. Apolipophorin-III mediates antiplasmodial epithelial responses in *Anopheles gambiae* (G3) mosquitoes. **PLoS One. 2010,** 5(11):e15410

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BRIEF BIO-DATA OF STUDENT

Ms. Dhawan has pursued Master's degree in Biotechnology from Amity Institute of Biotechnology, Amity University, Noida. She qualified CSIR NET-LS with an All India rank of 32. She has also qualified the GATE-2012 examination. She joined BITS-Pilani, Biological Sciences group as a PhD student under the mentor-ship of Dr. Lalita Gupta in Jan, 2012. During this period, she was appointed as Project Assistant in DST Fastrack Project "Characterization of STAT Signaling Pathways in *Anopheles*". Ms. Dhawan has presented 3 research papers in National conferences and 1 International Conference. She has published 9 research papers and three as first author during her PhD tenure.

Immune Responses of *Anopheles stephensi* and *Anopheles culicifacies* against Malaria Parasite Development

THESIS

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By

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CONCLUSION

There are approximately 3,500 species of mosquitoes grouped into 41 genera. Human malaria is transmitted only by females of the genus *Anopheles*. Of around 450 *Anopheles* species, only 30-40 transmit malaria in nature. Some *Anopheles* species are poor vectors of malaria, as the parasites do not develop well (or at all) within them. Besides, there also occurs variation within species. Vector and parasite relationships are extremely specific and peculiar in nature which determines the dynamicity of parasite development and malaria transmission among humans (Sinka et al., 2012). It has been hypothesized that the molecular mechanism of a variety of malaria vector species varies with pathogen which might have different due to disproportional susceptibility (Molina-Cruz and Barillas-Mury, 2014). Recently, a landmark study was published by Molina-Cruz et al., 2015 in which they have proposed the lock-key theory, which implies that with different haplotypes of Pfs47 gene of *Plasmodium* strains, the malaria vector species of different geographical region behave differently. Why only certain species or strains of Anopheline mosquitoes are major malaria vectors and what determines the vector competence remains an enigma and warrants its elucidation. Therefore, this brings forth the importance to detail the immune responses generated in different vector species due to pathogen invasion. In the current work, immune responses of two major malaria causing vector species, i.e., *Anopheles stephensi* and *Anopheles culicifacies* have been studied at molecular level. It will help to enhance our knowledge about vector-parasite interaction in mosquito species of Indian sub-continent where malaria is still a major problem.

Some of the major conclusions from the thesis are highlighted as follows:

- Detailed *in-silico* prediction of ApoLp-III gene in *Anopheles stephensi* as well as identification of ApoLp-III gene in the genome of recently sequenced 16 *Anopheles* species was carried out. The putative ApoLp-III from these Anophelines were comparatively analyzed among themselves as well as with other insects and their phylogenetic relationship had also been studied. The phylogenetic analysis revealed the clear divergence of the gene from their lepidopteron and other dipterans counterparts.

- Through cloning and sequencing of Apolipophorin-III gene in *An. stephensi* its precise sequence and introns-exons boundaries were determined. Its role during *Plasmodium* infection within the mosquito had been analyzed in details. AsApoLp-III mRNA expression was strongly induced in *Plasmodium berghei* infected mosquito midguts at 3h and 24h, suggested its significant role in parasite development.

- Functional characterization of ApoLp-III in *An. stephensi* was accomplished using RNA interference technique. It was seen that AsApoLp-III silencing decreased *P. berghei* oocysts numbers by 7.7 fold against controls. These effects might be due to the interruption of AsApoLp-III mediated lipid delivery to the developing oocysts. Earlier studies of different strains of *An. gambiae* (Yaounde' and G3) with *P. berghei* showed either no effect or opposite effect (Mendes et al., 2008 and Gupta et, al., 2009) suggesting each *Anopheles* species may respond differently with same parasite.

- Nitric oxide synthase (NOS), an antiplasmodial gene, was also highly induced in AsApoLp-III silenced midguts suggested that AsApoLp-III acts like an agonist which aids in the development of *Plasmodium* and protects it against the mosquito immunity.

- STAT pathway genes: the transcription factor STAT and the regulatory proteins SOCS and PIAS were characterized in another major Indian malaria vector *Anopheles culicifacies*.

- The STAT pathway genes were present in all developmental stages of mosquito with prominence at initial egg stages. Also, the q-PCR analysis hints towards the male biasness of these genes.

- The effect on expression of STAT pathway genes in midguts of *An. culicifacies* mosquito 24 hour after *P. berghei* infection was investigated. Quantitative PCR analysis revealed that AcSTAT-A mRNA levels were

remain unchanged during *P. berghei* infection, however, AcSOCS and AcPIAS, higher expression was observed in the infected midguts compared to blood-fed controls. This indicated that STAT pathway might be induced during ookinete invasion and in parallel the suppressors of this pathway were also activated to counter balance the immune reactions.

- Cloning and Characterization of the genes encoding ROS detoxifying enzymes (Catalase and SOD) were analyzed in *An. culicifacies* mosquitoes. Their mRNA expression in all developmental stages of mosquito suggested that both genes were required in all stages.

- The reduced expression of CuZnSOD in *An. culicifacies* at 24h post *P. berghei* infection suggested that upsurge intracellular levels of free radicals in the carcass (rest of the body except midgut) may help in the clearance of *Plasmodium* load.

- AcCatalase mRNA levels were induced in the infected midgut and carcass as compared to control. This might be the outcome of two prospects; either the mosquito tends to lower H_2O_2 generated as an immune response to normal levels in their body, a probable step to neutralize increased production by the activated immune system, or it might be a strategy of *Plasmodium* to modulate the immune response of mosquito.

FUTURE PROSPECTIVE

The scope of this thesis was to identify the important immune genes which were playing a crucial role in mosquito immune system against *Plasmodium* infection. The focus of the thesis was to decipher the immune response of different mosquito species against *Plasmodium* infection. A further research will include the functional elucidation of following future perspectives:

- ApoLp-III comprises of alpha helices arranged in up and down topology. It would be interesting to know the mode of action of this protein in mosquito hemolymph during pathogen interaction.

- It would be interesting to know the effect of silencing of AsApoLp-III on other immune genes to understand the molecular mechanism that aids in development of *Plasmodium* infection.

- ApoLp-III is a multi-functional protein and is found to be playing crucial roles in other insect species. The interaction of ApoLp-III with ApoLp-I/II in transfer of lipid during flight and also finding out the role of this gene in fecundity of mosquito is a subject of greater interest.

- After the establishment of their role in *Plasmodium* development, functional characterization of STAT pathway gene in *An. culicifacies* can be the future refinement of this thesis.

- Characterization and Expression analysis of ROS detoxifying enzymes genes in *An. culicifacies* against *Plasmodium* had been discussed. It would be interesting to functionally analyze these detoxifying genes during infection through RNAi.