The Identification and Characterization of Mosquito Peroxidase(s) and their role in orchestration of Mosquito Immunity

THESIS

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By

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CERTIFICATE

This is to certify that the thesis entitled **The Identification and Characterization of Mosquito Peroxidase(s) and their role in orchestration of Mosquito Immunity** submitted by **Tania Pal Choudhury** ID No **2013PHXF0426P** for the award of Ph.D. degree of the Institute embodies original work done by her under our supervision.

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Dedicated to the Almighty, my Parents,

and

My beloved Husband

ABSTRACT

Anophelines are notorious vectors of *Plasmodium* parasites that cause malaria and in the recent years there is an increase in cases reported worldwide with India contributing the 4th highest number of malaria cases and deaths (WHO report, 2017). Research in the past few decades has focussed on the exploration of strategies to control malaria in the vector itself, thus blocking the transmission of the parasite to human host. The emergence of drug-resistant parasites and insecticide resistance coupled with economic challenges as well as the implementation of control strategies are major hurdles in this area. Moreover, with a renewed emphasis on global eradication of malaria, the genome-based discovery of novel anti-transmission candidates have been identified as one of the priority research areas for the immediate future. In particular, the interactions between the mosquito immune system and/or midgut microbiota and its pathogens are critical determinants of the outcome of pathogen infection and transmission.

There are two branches of immunity in mosquitoes: the humoral and the cellular immunity, and pathogens face an intricate challenge against the cascade of response elicited by a systematic and concerted approach by these two branches. While the *Plasmodium* parasite interacts with the midgut environment upon entry, with the response being mainly humoral in nature initially, the bacteria, virus or fungi encounter both cellular and humoral responses upon their recognition. However, pathogens have evolved various strategies to bypass the mosquito immunity. In this regard, various mosquito molecules important for pathogen development and/or transmission have been identified through decades of research. Here, we have utilized the currently available whole-genome transcript microarray data, previous literature reports, and employed transcriptional analysis, RNAi-mediated gene silencing, as well as other tools to investigate the role of two heme peroxidases (HPXs) – DBLOX, a double peroxidase i.e. it has two heme peroxidase domains I and II (put the detail here) and HPX8 from an important Indian malaria vector - *Anopheles stephensi* in mosquito innate immunity. These findings highlight the non-canonical antibacterial defences of mosquito innate immunity that could be exploited to interrupt pathogen transmission.

In this thesis work, we carried out the molecular characterization of heme peroxidase gene- DBLOX. The gene is expressed in all stages of *An. stephensi* development, with maximum expression in the pupa stage. The AsDBLOX gene was

also induced by bacterial challenge in both the larvae and adults of *An. stephensi*. Also, its silencing caused an increase in bacterial population.

It's *in silico* analysis revealed that AsDBLOX gene has two integrin binding motifs in HPX domain I that otherwise has only substrate binding sites present in it and lacks heme and calcium binding sites. The HPX domain II, on the contrary, has all the requisite binding sites making it a complete functional domain. Also, we analyzed the selection pressures acting on the two domains of AsDBLOX.

Further, we characterized another heme peroxidase from *An. stephensi*- AsHPX8. It is a secreted protein and has heme binding, calcium binding and substrate binding sites characteristic of heme peroxidase proteins. The AsHPX8 gene is induced in mosquito blood fed midguts as well as exogenous bacteria fed with blood/saline. This indicated that AsHPX8 is one of the genes that shows significant differential expression upon pathogen challenge in the midgut of female *An. stephensi* mosquitoes.

In addition to that, it also has a role in mosquito development. Insects are reported to have a chorion peroxidase which is involved in chorion protein crosslinking i.e. hardening which makes them resistant to desiccation. We found that AsHPX8 gene is induced in blood-fed carcasses and based on previous reports of a chorion peroxidase induced post blood feeding; we assumed that AsHPX8 plays crucial role in chorion hardening. However, upon silencing of this gene, we observed no effect on number of eggs laid by the mosquitoes. Further analysis revealed the induction of its duplicated paralog AsHPX7 gene in the AsHPX8 silenced samples.

This thesis work contributes to our current knowledge of mosquito immunity and reproduction. While DBLOX plays crucial role in *An. stephensi* antibacterial immune responses, HPX8 functions a dual role by maintaining midgut homeostasis as well as an important role in mosquito life cycle. Hence, AsDBLOX and AsHPX8 are attractive targets which can be further explored for vector control measures.

Declaration

I hereby declare that this Ph.D. thesis entitled **"The Identification and Characterization of Mosquito Peroxidase(s) and their role in orchestration of Mosquito Immunity"** is my original work which was carried out for the degree of Doctor of Philosophy under the supervision of **Prof. Prabhat Nath Jha** (Head, Department of Biological Sciences, BITS Pilani, Pilani Campus) and co-supervision of **Prof. Sanjeev Kumar** (then Associate Professor, Department of Biological Sciences, BITS Pilani, Pilani Campus and currently Professor and Head, Department of Biotechnology,Ch. Bansi Lal University- CBLU, Bhiwani, Haryana) at Birla Institute of Technology and Science, Pilani, Pilani campus, India. I have duly acknowledged all the sources of information used in the work presented in the thesis.

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Abbreviations

HPX	Heme peroxidase
As	*Anopheles stephensi*
Ag	*Anopheles gambiae*
PBS	Phosphate buffer saline
FBS	Fetal bovine serum
PCR	Polymerase chain reaction
qPCR	Quantative PCR
gDNA	Genomic DNA
cDNA	Complementary DNA
mRNA	Messenger RNA
rRNA	Ribosomal RNA
NaCl	Sodium chloride
Tris-Cl	Tris(hydroxylmethyl)aminomethane-Hydrochloride
kDa	kilo Dalton
AA	Amino acids
bp	Base pair
dsRNA	Double stranded RNA
RT	Room temperature
TF	Transcription factor
AMPs	Anti-microbial peptides
ROS	Reactive oxygen species
DBLOX	Double Peroxidase
NOS	Nitric oxidase synthase
TEP1	Thioester-containing protein

Keywords

S. No.	Keywords
1	Mosquito
2	Malaria
3	*Anopheles gambiae*
4	*Anopheles stephensi*
5	Immunity
6	Midgut
7	Heme peroxidase (HPX)
8	Hemocyte
9	Peroxinectin
10	*Plasmodium*
11	Bacteria
12	DBLOX
13	HPX8
14	Chorion peroxidase

Chapter 1

Introduction and Review of Literature

1.1 Current malaria scenario across the world and in the Indian subcontinent

Mosquitoes are notorious vectors responsible for transmitting diseases like malaria, dengue, etc. and cause a huge impact on public health and socioeconomics. Their sheer abundance, compounded by their maneuverability and hematophagous (blood-feeding) nature, makes them excellent candidates as carriers of pathogens. Malaria, caused by the Apicomplexan parasite *Plasmodium*, affects approximately 3 billion people worldwide every year. The parasite is transmitted to its vertebrate host by the bite of a female *Anopheles* mosquito. There are more than 100 *Plasmodium* species and their vertebrate hosts range from mammals (primates and rodents) and birds (wild and domestic species) to reptiles (lizards and snake) (https://www.cdc.gov/malaria/about/biology/parasites.html). Out of these, only six species are reported to infect humans, viz. *P. falciparum*, *P. vivax*, *P. ovale, P. malariae* and recently reported *P. knowlesi* (William et al., 2013; White, 2008) and *P. cynomolgi* (Ta et al., 2014). In the year 2017, the global tally of malaria mortality incidences reached 445,000, which is about the same as reported in 2015 (WHO, 2017) (**Figure 1.1**).

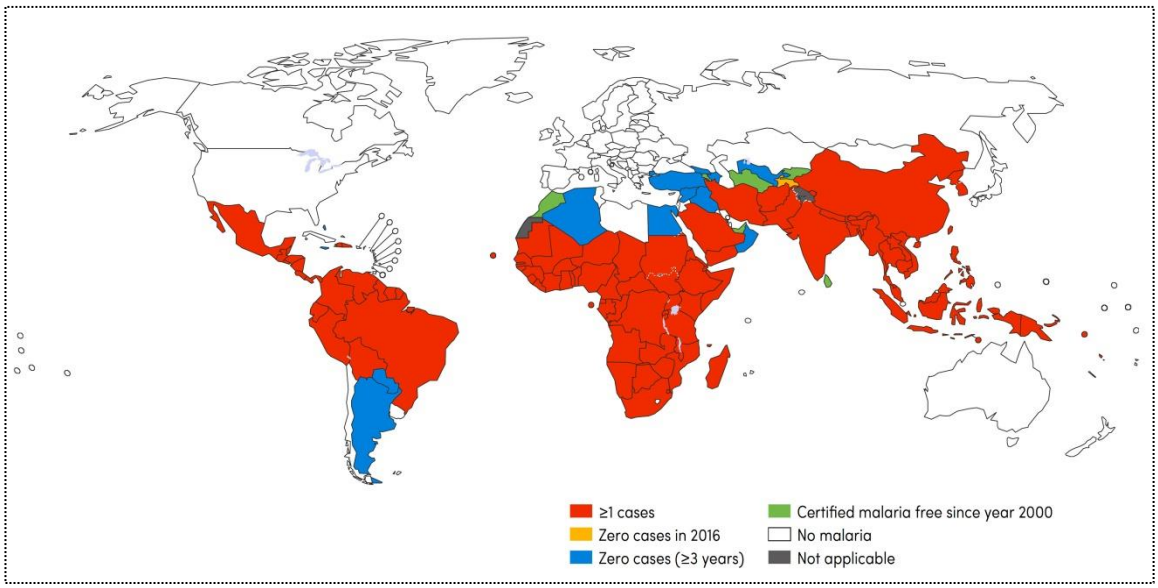


Figure 1.1: Worldwide distribution of malaria (WHO Malaria Report, 2017).

Figure 1.2 shows that worldwide, fifteen countries account for 80% of all malaria cases and India has the third highest number of malaria cases and deaths in the world (WHO, 2017). Moreover, malaria is endemic in nine of these fifteen countries, accounting for nearly 70% of the disease burden. Also, nearly 63% of the cases are due to *P. falciparum*. In the year 2016, India accounted for 80% of the reported cases and

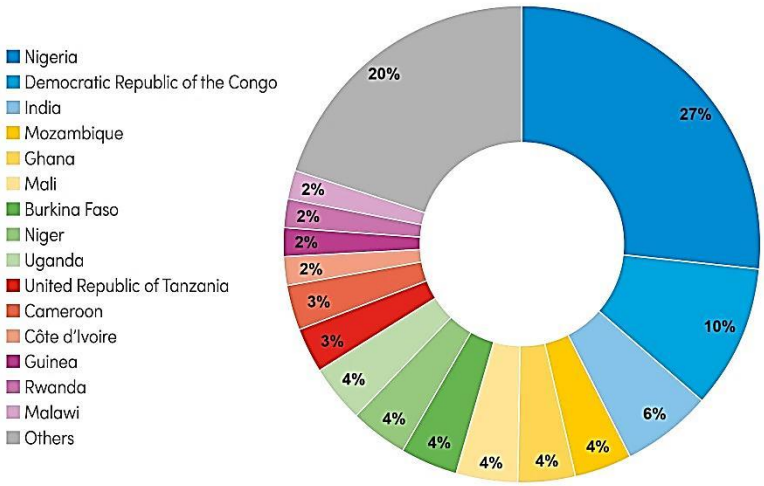


Figure 1.2: Country-wise estimated share of total malaria cases (WHO Malaria Report, 2017).

60% of the malaria mortality incidences (WHO, 2017). This recent data alarms the high percentage of the world population which is under risk of deaths due to malaria.

1.2 Life cycle of *Plasmodium* the malaria parasite

1.2.1 *Plasmodium* infection of the human host

Plasmodium in its entire life cycle, completes four asexual stages in humans i.e. the intermediate host (hepatic merozoites followed by intraerythrocytic trophozoites, schizonts, and gametocytes) and three sexual stages in mosquitoes i.e. the definitive host (sexual stages-; ookinetes, oocysts, and sporozoites). The bite of an infected female *Anopheles* mosquito releases ~100-200 sporozoites into the subcutaneous tissue of host (Medica and Sinnis, 2005) (**Figure 1.3).** The asexual life cycle begins with the bite of a female *Anopheles* mosquito. Some of these sporozoites make their way into the peripheral blood circulation infecting hepatocytes (Vanderberg and Frevert, 2004; Yamauchi et al., 2007; Amino et al., 2008) initiating the pre-erythrocytic cycle and releasing thousands of merozoites into the circulation within 6-12 days. Although some of the merozoites are phagocytosed, the others enter erythrocytes to begin the erythrocytic cycle (or erythrocytic schizogony) (Kumaratilake and Ferrante, 2000). In an erythrocyte, each merozoite develops through ring and trophozoite stage into schizont containing multiple merozoites (erythrocytic schizogony). Further, with the rupture of schizont, these merozoites are released into the bloodstream where they reinvade the fresh erythrocyte to perpetuate the erythrocytic cycle. The factors triggering gametogony are not clearly understood and may include an interplay between a number of host factors as well as parasite signaling pathways (Kawamoto et al., 1991; Billker et al., 1997; Garcia et al., 1998; Sologub et al., 2011).

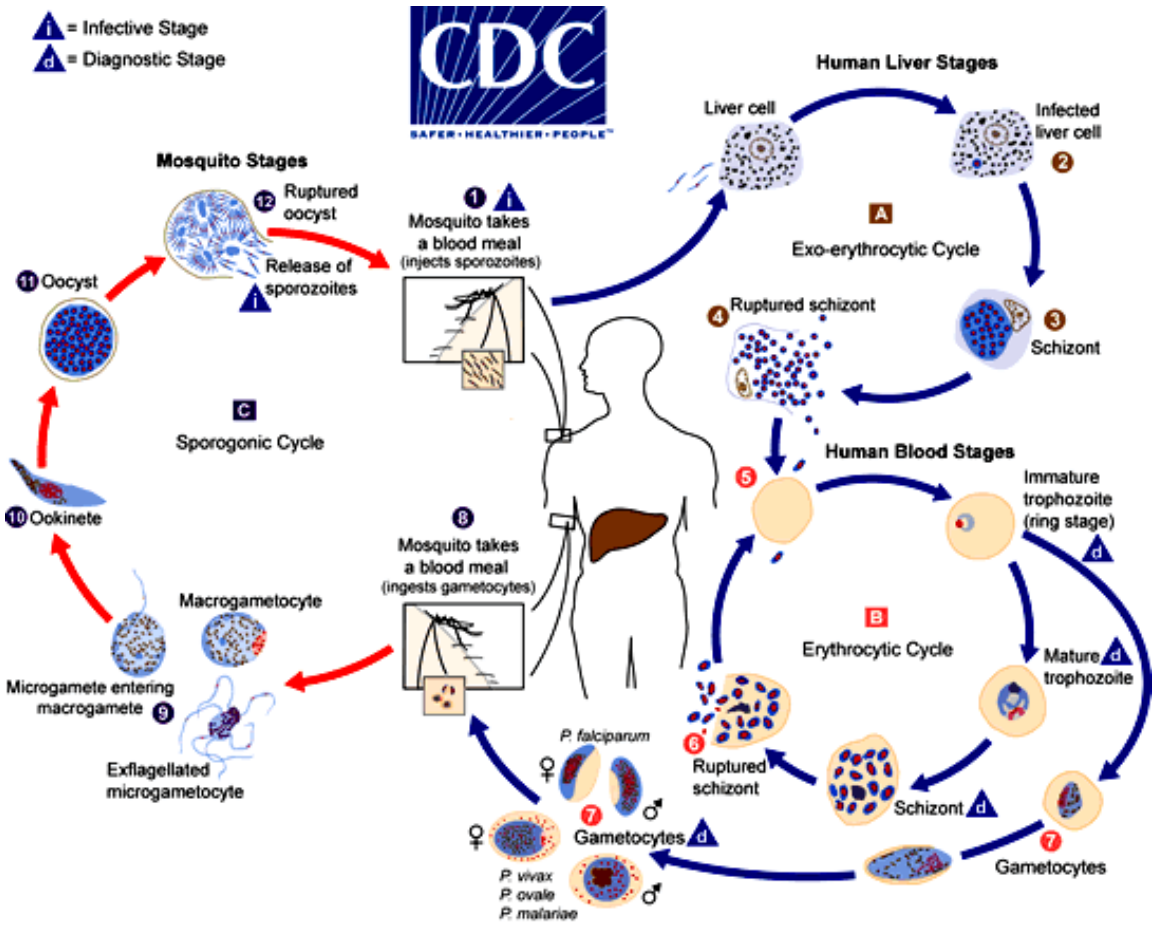


Figure 1.3: *Plasmodium* life cycle in two hosts: human and female *Anopheles* mosquito. It involves two hosts. During a blood meal, a *Plasmodium*-infected female *Anopheles* mosquito injects sporozoites into the human host (1). Sporozoites infect hepatocytes (2) and either enter a dormant hypnozoite state or mature into schizonts (3), which rupture and release merozoites (4). Post this initial replication in the liver (exo-erythrocytic schizogony A), *Plasmodium* undergo asexual multiplication in the RBCs (erythrocytic schizogony B). Merozoites infect RBCs (red blood cells) (5). The ring stage trophozoites mature into schizonts, which rupture, releasing merozoites (6). Some parasites differentiate into sexual erythrocytic stages (gametocytes) (7). Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes- male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal (8). The parasite multiplication in the mosquito is known as the sporogonic cycle (C). While in the mosquito's midgut, the gametocytes exflagellate and microgametes penetrate the macrogametes generating zygotes (9). The zygotes, in turn, transform into elongated motile ookinetes (10) which invade the midgut epithelium of the mosquito where they settle themselves under basal lamina and develop into oocysts (11). The oocysts grow, rupture, and release sporozoites into the mosquito hemocoel (12), which make their way to the mosquito's salivary glands. Inoculation of the sporozoites (1) into a new human host perpetuates the malaria life cycle. Source: Center for Disease Control and Prevention (CDC, Atlanta) March, 2018.

1.2.2 *Plasmodium* infection of *Anopheles* mosquito

Plasmodium development in mosquito involves primarily three tissues- i. midgut (male and female *Plasmodium* gametocytes in an ingested blood meal, develop into male microgametes and female macrogametes, respectively, which then exflagellate and fertilize into zygotes. Zygotes then transform into motile ookinetes which invade and migrate across the midgut epithelia, in ~15 h after the ingestion of an infected blood meal. Once the diploid ookinetes reach the midgut epithelia's basal side by ~24 h, they

transform into ovoid oocysts, which then undergo multiple rounds of replication by sporogony in ~10 d), ii. Hemocoel (10-12 days post blood meal, oocysts containing thousands of haploid sporozoites are ruptured, which are then released into the mosquito hemocoel at about 14 days after the blood meal and migrate through the mosquito hemolymph in order to invade the third tissue) iii. salivary gland (sporozoites undergo further maturation and are subsequently injected with saliva into human host upon blood feeding) (Sinden, 2002).

1.3 Malaria vector distribution

Presently, there are approximately 3500 species of mosquitoes recognized worldwide and, all of them belong to the monophyletic family of Culicidae. They are distributed among the three subfamilies Culicinae, Anophelinae, and Toxorhinchitinae. Even

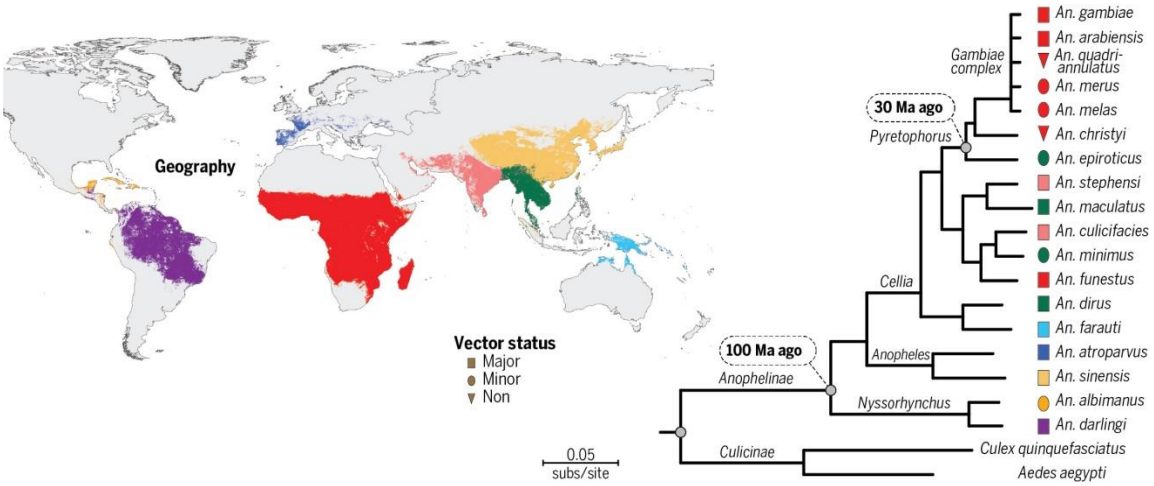


Figure 1.4: Geography, vector status, and molecular phylogeny of the 16 newly sequenced anopheline mosquitoes and selected other dipterans (Neafsey et al., 2015).

though they share many characteristics, there exists considerable ecological and physiological diversity within the family. The subfamily Anophelinae is the most ancestral group that radiated in Africa and South America approximately 100 million years ago (Krzywinski and Besansky, 2003), and possess a relatively large number of species complexes whose sibling species are morphologically indistinguishable but genetically distinct (White, 1974; Collins and Paskewitz, 1996). Of the approximately 465 *Anopheles* species across the world, merely 41 are considered as vectors of malaria (Sinka et al., 2012). These anophelines are distributed worldwide as shown in **Figure 1.4** and transmit different species of *Plasmodium* depending on the region and the local environment.

Moreover, tropical countries like India, favor the growth of mosquitoes (Anvikar et al., 2016). Of the 58 anophelines reported from the Indian subcontinent, only six taxa are major malaria vectors (**Figure 1.5**). **Table 1.1** shows the major malaria vectors in India with the reported cases and number of districts (Dev and Sharma, 2013;). All the *Anopheles* species referred above except *An. stephensi* have been characterized into species complexes with morphologically distinguishable forms.

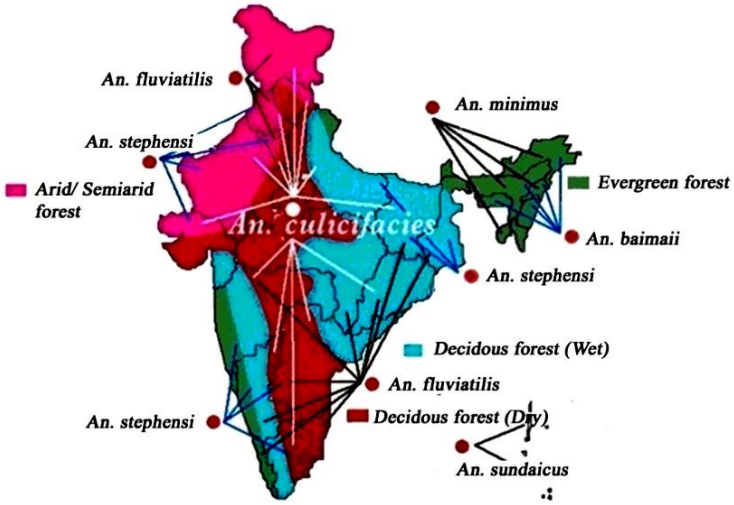


Figure 1.5: *Anopheles* vectors in India.

Table 1.1: *Anopheles* vectors in India, their preferred habitat, and % (population) reported cases and districts in India (Dev and Sharma, 2013; Singh et al., 2017).

Sl. No	*Anopheles* species	Localization	Reported cases	Reported no. of districts
01	*An. culicifacies* s.l.	rural	65%	421
02	*An. fluviatilis* s.l.	plains and foothills	15%	241
03	*An. dirus* s.l.	jungles of northeastern states	-	-
04	*An. minimus*	foothills of the northeast	-	-
05	*An. sundaicus*	Andaman and Nicobar islands and breeds in brackish water	-	-
06	*An. stephensi*	urban	-	243

1.4 Life cycle of *Anopheles stephensi*

Mosquitoes are holometabolous insects, i.e., complete metamorphosis in the life cycle. Their life cycle consists of four stages: eggs (laid in water and are prone to desiccation), four larval instars (LI, LII, LIII, LIV- aquatic in nature, voracious feeders), pupae (aquatic, non-feeding) and adult males and females (aerial). It takes ~2 weeks for completion of their life cycle (https://www.cdc.gov/malaria/about/biology/mosquitoes/index.html). Also, *An. stephensi* prefers human blood over other hosts (Swami and Srivastava, 2012).

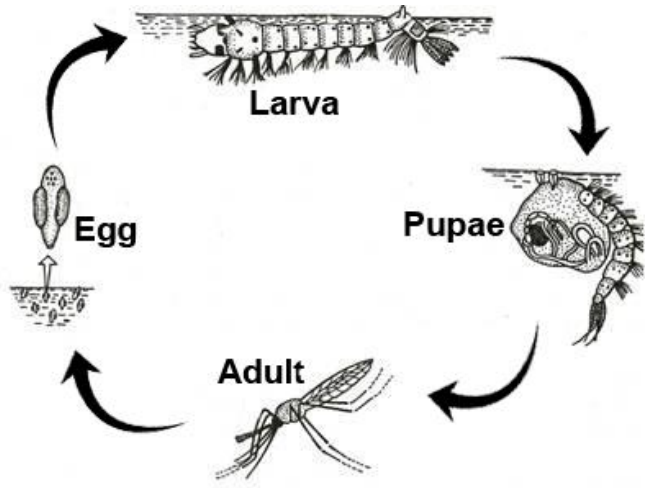


Figure 1.6: Mosquito life cycle. The life cycle of *Anopheles* starts with an egg that hatches into larva. The larva undergoes three consecutive moults and transforms into fourth instar larva. Larva finally develops into pupae, metamorphosis takes place and develops into an adult (WHO, 1997, Vector Control Methods for Use by Individuals

1.5 Malaria treatment and prevention strategies

The approach is twofold. First employs drugs and vaccines targeting the human stages of *Plasmodium*, and the second targets the vector stages of the parasite. While the first approach aims directly in the treatment of a malaria-infected patient, the second one is a

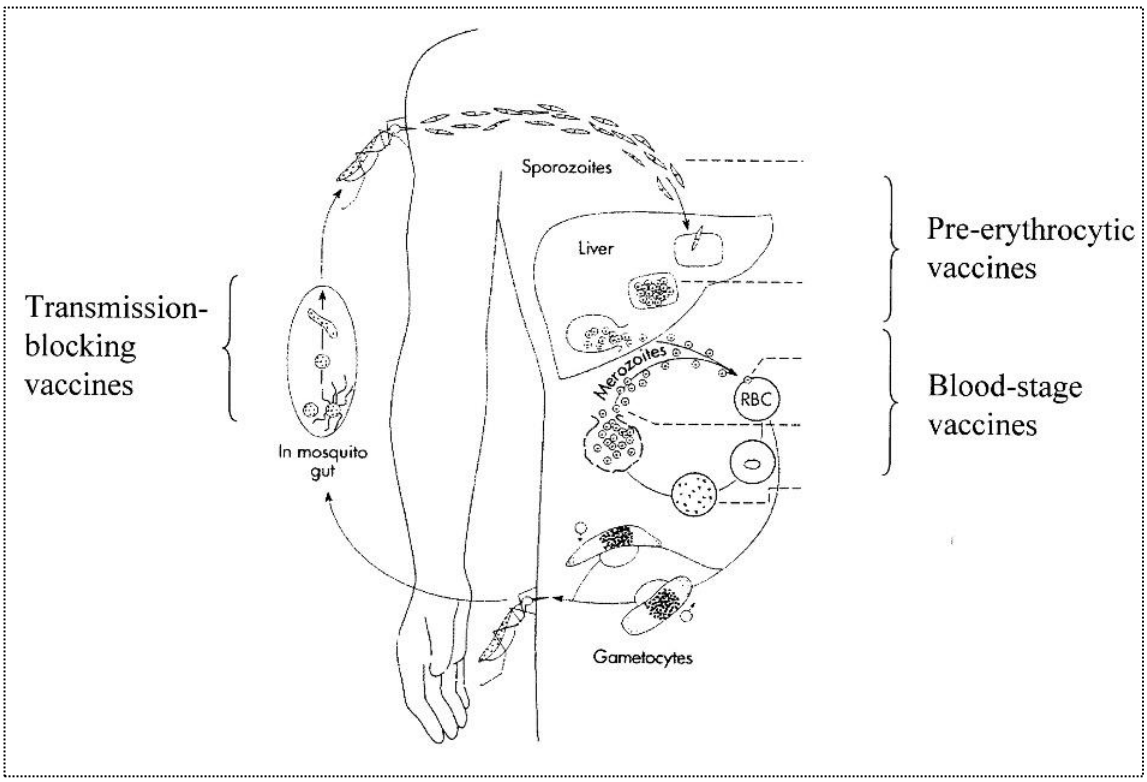


Figure 1.7: *Plasmodium* life cycle and types of anti-malarial vaccines. Pathways and strategies for developing a malaria blood-stage vaccine (Good et al., 1998).

more indirect method of curbing malaria, by reducing the transmission of *Plasmodium* by *Anopheles*, thus reducing the chances that other individuals in that community contract malaria. Over the course of time, the latter approach would lower the probability that an immunized individual would contract malaria again. In this regard, vaccines have

been reported to be promising targets for malaria control and are viewed by some experts to be necessary for successful malaria elimination

Vaccines are classified into three types- i. pre-erythrocytic vaccines: against sporozoite and liver-stages; ii. blood-stage vaccines: clear parasitemia and prevent clinical disease; and iii. transmission-blocking vaccines: prevent infection of mosquitoes and interrupt malaria transmission in populations (**Figure 1.7**) (Hill 2011). Nowadays, there is emerging appreciation for vaccines combining multiple targets and stages. For instance, the vaccine RTS,S/AS01 targets the pre-erythrocytic stages of *P. falciparum* malaria in children and is the first vaccine that successfully completed a Phase III clinical trial (Duffy et al., 2012).

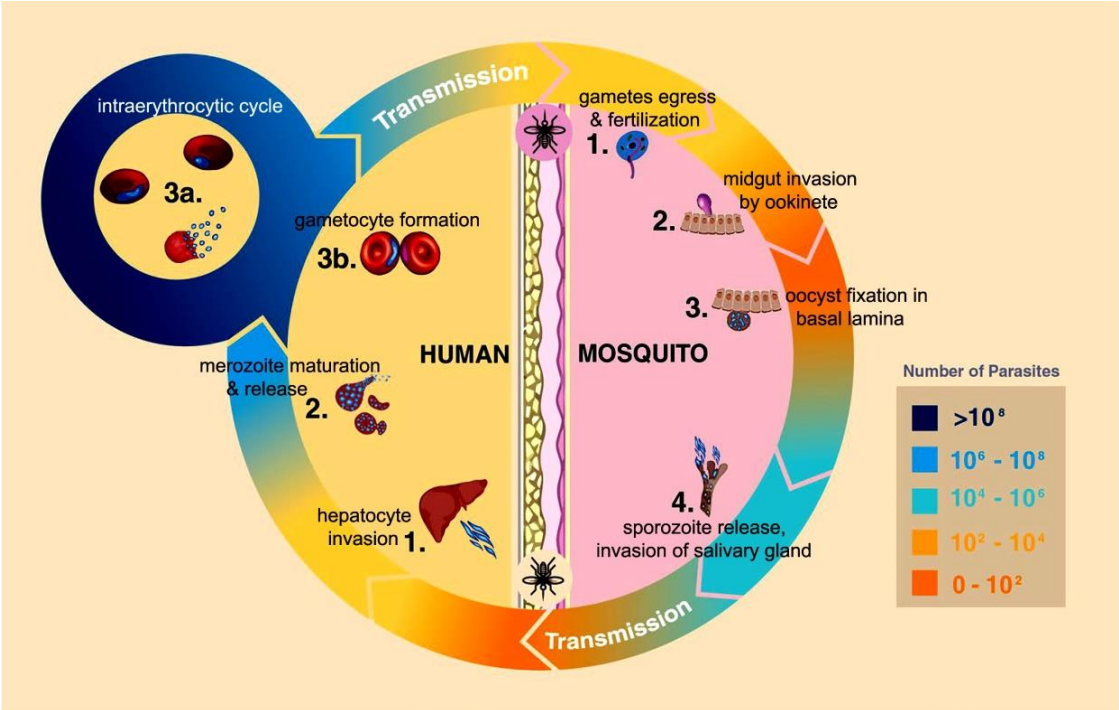


Figure 1.8: *Plasmodium* parasite bottleneck in *Anopheles:* The parasite numbers fluctuate during its sexual stages of life cycle (Smith et al., 2015).

Pre-erythrocytic and blood stage vaccines: The pre-erythrocytic (PE)-stages *Plasmodium* parasite are attractive vaccine targets for several reasons, like a. very low number of infected hepatocytes, b. *Plasmodium falciparum* and *Plasmodium vivax* take nearly a week for their development in hepatocytes, providing sufficient time for elimination and c. the infected hepatocytes are capable of presenting parasite antigens to immune effector cells. In this regard, several proteins are proposed as vaccine targets, for instance immunization with CelTOS, a micronemal protein and essential component of cell traversal machinery, induced humoral and cell-mediated immune responses against *Plasmodium yoelii* with cross-species activity against *Plasmodium berghei* (Kariu et al., 2006). Other examples are proteins important for cell

traversal like (SPECT and SPECT2) and phospho-lipase (Ishino et al., 2004; Ishino et al., 2005). Other candidates target merozoites and blood-stage parasites- Erythrocyte Binding Antigen (EBA-175), Merozoite Surface Antigen 1&2 (MSA-1&2), Apical Membrane Antigen-1 (AMA-1), etc. (Cortés et al., 2007)

Transmission-blocking strategies (TBS) : There are three main TBS that are being pursued- a. gametocytocidal drugs (Pukrittayakamee et al., 2004; Pukrittayakamee et al., 2008; Delves et al., 2012), b. transmission-blocking vaccines (TBV) (Carter, 2001; Willyard, 2018) and c. shifting mosquitoes towards refractory traits (Hammond et al., 2016). A TBV given to an entire community especially at one point in time could drastically reduce the number of parasites traveling through the mosquito and back into another person. Over time, the numbers of mosquitoes and people carrying the parasite in a given community would decline which would, in turn, steadily reduce the number of new malaria infections in that community and lead to “herd immunity.” A TBV could become part of a comprehensive elimination and eradication effort by helping to drive parasite transmission to zero - especially in settings where other interventions have already had an impact. The immunity induced by a TBV would also be independent of user behavior even in the most challenging environments and no matter where the immunized person goes. Such a vaccine could serve as a kind of “safety net,” protecting populations whose natural immunity may have waned upon exposure to parasites. Hence, it is important to have new medicines available that are active against emerging resistant strains of the parasite.

TBVs against *Plasmodium* stages in mosquitoes - The antigens such as 1. Pfs230, Pfs48/45 (present on the surface of *P. falciparum* gametocytes and gametes), 2. Pfs25/Pfs28 (present on the surface of parasite zygote and ookinetes) have been successfully targeted through transmission-blocking vaccines (Chaturvedi et al., 2015).

TBVs targeting *Anopheles* innate immunity molecules - Manipulation of mosquito innate immunity has been touted as a possible approach to negatively affect their capacity to serve as parasite hosts. New TBS being studied differ mainly from the classical vector control approaches, such as the use of insecticides, because they are designed for mosquito survival, thus avoiding selective pressure towards resistance (Ramirez et al., 2009; Nwane et al., 2013). In the mosquito, midgut, hemocoel, and salivary glands are tissues which interact with *Plasmodium*. Hence, if parasite transmission is interrupted in any or all of these compartments by genetically modifying the expression of these “mosquito genes,” then we can inhibit or reduce parasite development.

Previously, *Anopheles* molecules like carboxypeptidase B (CPB), aminopeptidase N (APN) and salivary gland antigens such as saglin and SGS1 (salivary gland surface protein 1) have been reported to play an important role in the development of *P. falciparum* inside the mosquito and, therefore, are considered as TBV candidates (Lavazec et al., 2007; Raz et al., 2013; Bokharaei et al., 2012; Armistead et al., 2014; VenkatRao et al., 2017; Okulate et al., 2007; Korochkina et al., 2006). These candidates are conserved in anophelines and elicit transmission reducing antibodies in mosquitoes (Dinglasan et al., 2007; Mathias et al., 2012). Promising results have been obtained from the research conducted in this area and hence, the screening of such new molecules will help in the development of more novel approaches. Further, this area needs understanding of the details of immune interactions between *Plasmodium* and mosquito immunity. Past evidence suggests that this strategy works successfully in the laboratory (Ito et al., 2002; Marrelli et al., 2007; Corby-Harris et al., 2010; Isaacs et al., 2011). But one huge challenge is how to drive transgenes into wild mosquito populations. Various gene drive mechanisms like CRISPR–Cas9 system have been proposed to accomplish this goal (Chen et al., 2007; Windbichler et al., 2011). Gene drives have tremendous potential for controlling disease vectors by altering genes in ways that reduce the insect population size or prevent them from spreading a parasite. These mosquitoes have tremendous potential to help eliminate malaria, and multiple gene drive approaches have recently shown promise in laboratory settings. These approaches include population suppression through fertility disruption, driving-Y chromosomes, and population replacement with genes that limit malaria transmission.

The mosquito's innate immune system has been shown to play a key role in killing parasites and thereby affecting parasite development. Mosquitoes, like other insects, are known to mount potent immune responses against invading bacteria, fungi, viruses and parasites (Lehane et al., 2004). Several pieces of evidence suggested that mosquito is able to trigger a series of diverse defense reactions upon *Plasmodium* infection. These defense mechanisms are believed to account for the reduction in parasite number (**Figure 1.8**). The details of immune responses of mosquitoes are discussed in the next section.

Other strategies: Use of insecticide-treated bed nets, repellents, insecticides, larvicides, and bacteria that produce toxins. Another strategy is to control vector population by interrupting their life cycle (vector control strategies).

1.6 Mosquito immunity

The arsenal of immune molecules in mosquitoes consists of two separate arms- the humoral and cellular immunity. While the humoral immunity employs the use of various soluble protein factors present in the hemolymph or plasma milieu of the mosquito, the cellular branch uses blood cells or hemocytes that are suspended in the hemolymph. These two branches and the key players involved therein are described briefly below:

1.6.1 Humoral immunity

The invasion of a pathogen into the hemocoel of a mosquito triggers humoral factors like prophenoloxidase (PPO), pattern recognition receptors (PRRs), serine proteases and opsonins which ultimately lead to melanization or phagocytosis (Nakhleh et al., 2017; Blandin and Levashina, 2007). Various methods of hemocytes activation against pathogens are reported, for example - lectins produced upon injury circulate in the hemolymph and bind to carbohydrate moieties in the cell walls of the pathogen and signal the recognition and mobilization of hemocytes, or the hemocyte chemotactic peptide (HCP) from the moth *Pseudaletia separata* (Klowden, 2007; Nakatogawa et al., 2009).

In addition, factors in the hemolymph are induced upon microbe invasion, and act as signaling molecules- for example, *Drosophila* spaetzle (Spz) is induced by PRRs like peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs) in the presence of bacteria and fungus. It, acts as a signaling molecule by binding to the Toll receptors on the hemocytes to initiate the production of transcription factors such as nuclear factor κ B (NF κ B) to induce transcription of various anti-microbial peptides (AMPs) (Dostert et al., 2005; Pal and Wu, 2009; Valanne et al., 2011) Imler et al., 2005; Uvell et al., 2007; Charroux et al., 2010). On the other hand, the **(Immune deficiency)** (IMD) pathway in *Drosophila* is elicited against Gram-negative bacterial infection, resulting in the activation of PGRPs which stimulate the intracellular IMD molecules (Hetru and Hoffmann, 2009). This triggers the JNK pathway (stress response and wounding) or the DREDD (dorsal-related immunity factor death related ced-3/Nedd2-like protein) which results in cleavage of Relish, a transcription factor that is responsible for inducing AMP production (Kleino et al., 2005; Hetru and Hoffmann, 2009).

In *An. gambiae*, AMPs are primarily produced in the hemocytes, fat body and occasionally anterior midgut after bacterial challenge (Levashina, 2004). The AMPs reported in mosquitoes are – defensins, cecropins, and gambicins (Vizioli et al., 2001b; Vizioli et al., 2000; Vizioli et al., 2001a). **Defensins** are primarily active against Gram-

positive bacteria, and rarely against Gram-negative bacteria (Richman et al., 1996; Bartholomay et al., 2004). **Cecropins** are 4 kDa peptides and were the first immune peptides characterized from insects (Steiner et al., 1981). They are reported to be induced in the fat body of bacteria challenged *An. gambiae* mosquitoes (Vizioli et al., 2000). **Gambicin**, on the other hand, is reported to be targeted against Gram-positive as well as Gram-negative bacteria, fungi and *Plasmodium berghei* (Vizioli et al., 2001a).

Apart from these, a crucial molecule of humoral branch of immunity in mosquitoes is thioester-containing protein (TEP). It is an immunoglobulin-like molecule and acts as an opsonin that assists in phagocytosis of pathogens. The *An. gambiae* genome contains 19 TEP gene homologs (*AgTep* 1–19). The AgTEP1 is secreted as a hemolymph protein with a size of 165 kDa (TEP1-full) and its cleavage results in the formation of an 80 kDa active fragment (TEP1-cut) (Levashina et al., 2001a). The TEP1-cut circulates in the hemolymph in association with two leucine-rich repeats (LRRs) proteins, LRIM1 and APL1C (Fraiture et al., 2009; Moita et al., 2005). These two LRRs act as TEP regulators and promote pathogen recognition as well as their clearance by TEP1 mediated lysis. TEP3, TEP4, LRIM1, fibrinogen-related proteins (FBN8), and *An. gambiae* Down syndrome cell adhesion molecule gene (AgDSCAM) are other reported PRRs (Dong et al., 2006; Lombardo et al., 2013; Moita et al., 2005).They are discussed in detail below.

1.6.2 Cell-mediated immunity

Hemocytes are the principal mediators of the invertebrate cellular immunity where an adult female mosquito is estimated to have ~500 - 4,000 hemocytes (Christensen et al., 1989; Hillyer and Strand, 2014). They are classified mainly into three cell types based on morphology and function – a. granulocytes: constitute 80-95% of adult hemocytes population and are highly phagocytic; b. oenocytoids: constitute ≤10% of all hemocytes, are non-phagocytic and are a major source of PPO (prophenoloxidase); c. prohemocytes: the remainder of the hemocyte population in adults, are proposed to be the hemocyte stem cell lineage in adult mosquitoes (Castillo et al., 2006); (Strand, 2008a; Hillyer and Strand, 2014; Rodrigues et al., 2010). In naïve adult mosquitoes, the majority (~3/4th) of the hemocyte population circulate freely in the hemolymph while the rest remain adhered to various abdominal organs like midgut and tracheae (Hillyer and Strand, 2014).

However, upon infection, this scenario gets altered, for example, it was found that infection with *Plasmodium berghei*, recruits hemocytes to the mosquito midgut and heart with a few of them circulating freely in the hemolymph (Rodrigues et al., 2010; Volz et al., 2005; King and Hillyer, 2012). This property of adhesion has led to difficulties in their specific isolation and thus is a limiting factor in the investigation of mosquito cellular immunity, whereas the other branch viz. humoral immunity is more elaborately elucidated, as it involves soluble molecules.

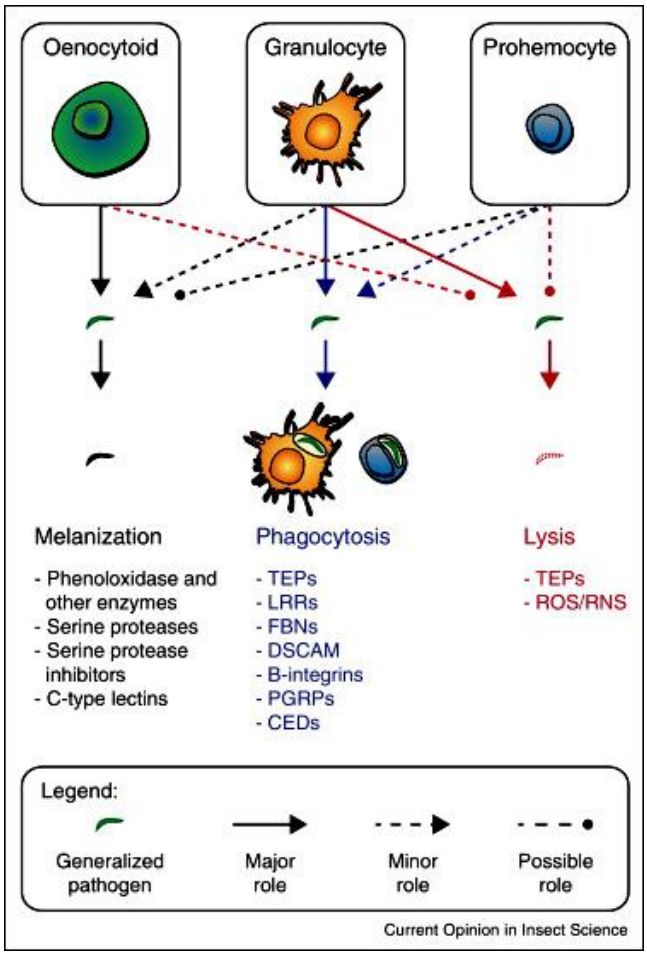


Figure 1.9: Hemocyte effector responses against various pathogens in mosquito immunity (Hillyer and Strand, 2014)

HEMOCYTE EFFECTOR RESPONSES

Insect hemocytes are responsible for important immune processes like clotting (or coagulation), phagocytosis and encapsulation of pathogens (Strand, 2008b). They also contribute to humoral responses by producing antimicrobial peptides, opsonins, and components of the melanization cascade. In anophelines, hemocytes engulf malaria sporozoites (Hillyer et al., 2007), bacteria (Levashina et al., 2001b) and are associated with melanotic capsules (Hernández-Martínez et al., 2002). Interestingly, hemocytes also express and secrete several key agonists and antagonists of malaria parasite development (Castillo et al., 2006; Blandin et al., 2004; Frolet et al., 2006; Volz et al., 2005; Abraham et al., 2005).

Clotting:

The closing of external wounds in the exoskeleton to prevent loss of excess hemolymph by clotting reaction is called coagulation (Theopold et al., 2004). The process starts by lysis of granulocytes at the site of wound followed by the deposition of

melanin and binding of additional hemocytes resulting finally into the closure of the epidermis (Lai et al., 2001).

Phagocytosis:

In insects, hemocytes are involved in phagocytosis, encapsulation and nodulation immune responses. It is an evolutionarily conserved process exhibited against bacteria, fungi, yeast, protozoa and even against cell debris and apoptotic cells generated during embryonic development and adult stages (Strand et al., 2008). Upon recognition by dissolved PRRs or cell surface PRRs, the foreign body is internalized into a phagosome which fuses with a lysosome and is hydrolytically digested. In contrast, encapsulation and nodulation involve the irreversible binding of multiple hemocytes to the surface of large foreign objects or bacterial aggregates, respectively, to form a multicellular sheath (Satyavathi et al., 2014). Mainly granulocytes and to a lesser extent prohemocytes, are highly phagocytic and initiate phagocytosis within 5 min of pathogen exposure (King and Hillyer, 2013; Hillyer et al., 2003a; Hillyer et al., 2003b) and some of them can phagocytose hundreds of bacteria within 24 hours of infection (Hillyer et al., 2007). Phagocytosis is effective in the sequestration of bacteria, yeast, *Plasmodium* and small inanimate particles (Da Silva et al., 2000; Hillyer et al., 2003a; Hillyer et al., 2007; Hillyer et al., 2004; Hillyer et al., 2003b).

Pattern Recognition Receptors for phagocytosis:

Transmembrane receptors expressed on the hemocyte surface like β integrin (BINT2), a peptidoglycan recognition protein (PGRP), a low-density lipoprotein receptor-related protein (LRP1), and a protein containing both zinc finger and LITAF domains (Lombardo et al., 2013; Moita et al., 2005) function as PRRs. These factors may recognize pathogens directly or recognize pathogens after they have been opsonized by humoral factors. Finally, the intracellular proteins- CED2, CED5, and CED6 regulate the internalization of pathogens. TEP1-mediated, TEP3-mediated, LRIM1-mediated and LRP1-mediated phagocytosis occurs through the CED6 pathway, whereas TEP4-mediated and BINT2-mediated phagocytosis occurs through the CED2/CED5 pathway (Moita et al., 2005).

1.6.3 Mosquito immune signaling pathways

Innate immunity has been extensively studied in *Drosophila melanogaster* (Hoffmann et al., 1999; Liu et al., 2016); (Mussabekova et al., 2017). There are three major signaling pathways reported in mosquito immunity: the Toll, the Immune deficiency (Imd), and the

Janus Kinase-Signal Transducers and Activators of Transcription (JAK-STAT) pathways (Christophides et al., 2002; Meister et al., 2005; Lemaitre et al., 1996).

The **Toll/NFκB pathway** activates antimicrobial peptide (AMP) synthesis against bacteria and fungus in *Drosophila* and against Gram-positive bacteria, fungi, viruses and *P. berghei* in mosquitoes (Lemaitre et al., 1996; Ramirez and Dimopoulos, 2010; Frolet et al., 2006). The **IMD pathway** is elicited by Gram-negative bacteria and *P. falciparum* (Lemaitre et al., 1995). As described earlier, the PRRs activate these pathways. The IMD pathway also regulate the above mentioned AgDscam, APL1, APL2, LRIM1, FBN9 and TEP1 (Meister et al., 2005; Riehle et al., 2008; Povelones et al., 2009; Blandin et al., 2004; Frolet et al., 2006; Garver et al., 2009 and 2012).

Non-self-molecules upon recognition, trigger proteolytic cascades which play a major role in insect innate immunity. Examples range from PPO (prophenoloxidase) pathway in insects, thioester-containing protein (TEP) in *Drosophila melanogaster* as well as hemolymph coagulation cascade in horseshoe crab (Söderhäll, 1982; Söderhäll et al., 1994); (Lagueux et al., 2000; Tzou et al., 2002);(Aoun et al., 2011; Kawabata et al., 1996; Beisel et al., 1999).

The *An. gambiae* **JAK/STAT-pathway** and LPS-induced TNFα transcription factor (LITAF)-like 3 (LL3) are also reported to mediate late phase immunity. The JAK/STAT pathway regulates expression of nitric oxide synthase (NOS) that has a negative effect on developing oocysts (Gupta et al., 2009). In the *An. gambiae*, LL3 binds to the promoter region of an anti-plasmodial gene named serine protease inhibitor 6 (serpin 6) and modulates its expression. Silencing of LL3 gene restricts the differentiation of hemocytes and their response to parasite infection that results in the increased number of oocysts in silenced mosquitoes. Thus, LL3 implicates late-phase immunity against *Plasmodium* oocysts through hemocytes (Smith et al., 2012 and 2015).

Although, there has been vigorous research in the past few decades, yet there remain several unexplored avenues of mosquito immunity to exploit against the malaria parasite. Another aspect of approach studied nowadays involves the mosquito endogenous midgut microbiome that is discussed in detail in the next section.

1.6.4 Role of gut microbiota in vector competence

Mosquitoes are exposed to bacteria throughout their life cycle, and they live in close association with symbiotic bacteria. While the larval and pupal gut harbor Photosynthetic *Cyanobacteria*, the adult midgut is predominated by *Proteobacteria* and *Bacteroidetes*

with core taxa of *Enterobacteriaceae* and *Flavobacteriaceae* (Wang et al., 2011).

These bacteria perform important functions in supplying nutrients, helping in host development, food digestion, reproduction and also defend the host against colonization by opportunistic pathogens (Kajla et al., 2015). The role of bacteria in blood digestion is evident by the secretion of hemolytic enzymes by *Serratia* and *Enterobacter* (Gaio et al., 2011). In *An. stephensi,* vitamin supplements are provided by the bacterial species *Asaia bogorensis* (Crotti et al., 2010).

The immune system is not triggered against these endosymbionts, as IMD pathway is reported to maintain this fine balance in *An. gambiae* and *D. melanogaster* (Ryu et al., 2008; Lemaitre and Hoffmann, 2007; Clayton et al., 2013). Upon infection, the detection of PAMPs by mosquito PRRs suppresses the negative regulator Caudal, hence activating the full-fledged immune response (Muyskens and Guillemin, 2008; Ryu et al., 2008; Leulier and Royet, 2009; Charroux and Royet, 2010; Royet et al., 2011). Mosquito midgut synthesizes a non-cellular chitinous peritrophic matrix (PM) in response to blood feeding (Moskalyk et al., 1996). It separates the midgut epithelia from the ingested blood. The midgut bacteria of the insect have also been shown to modulate vectorial capacity by preventing the development of pathogens. The symbiotic bacteria in the midgut activate the basal innate immune activity through induction of ROS producing gene, AMPs and other immune-specific genes that can also act against *Plasmodium* (Cirimotich et al., 2010). Moreover, immune responses raised by the mosquito against bacteria and *Plasmodium* overlaps to a large extent. Additionally, host immune responses raised against endogenous gut bacteria also affect *Plasmodium* development (Dimopoulos et al., 2002).

This is an emerging area of interest since different *Anopheles* strains harbor quantitatively and qualitatively different microbial flora. The microbial environment can influence *Plasmodium* development within the mosquito (Pumpuni 1996). Hence, these studies highlight the potential for exploiting microbial functions for symbiotic control of mosquito-borne diseases (Ricci et al., 2012).

Thus, it can be concluded that midgut bacteria play a role either directly through the production of various enzymes and toxins or indirectly by stimulating the mosquito's innate immune system to produce antimicrobial molecules that limit *Plasmodium* development (Pumpuni et al., 1993; Azambuja et al., 2005; Dong et al., 2009a). The mechanisms by which *Anopheles* midgut maintains bacteria homeostasis and process of bacteria-mediated inhibition of *Plasmodium* infection are studied only to a limited

extent. Hence, these studies highlight the potential for exploiting microbial functions for symbiotic control of mosquito-borne diseases (Ricci et al., 2012).

1.7 Immune role of heme peroxidases

A heme peroxidase performs two different types of reactions. One is the formation of dityrosine covalent bonds via peroxidase cycle (dehydrogenation) in which H_2O_2 is reduced to water, and one-electron donors such as oxygen are oxidized to the respective superoxide radical. The other reaction is catalyzed by chloro-peroxidase which involves the oxidation of two-electron donors like halides (X^-) to the corresponding hypohalous acids (HOX) (Zámocký et al., 2015). Zamocky et al. (2015) reported the independent evolution of four heme peroxidase superfamilies, namely (1) peroxidase–catalase superfamily, (2) peroxidase-cyclooxygenase superfamily, (3) peroxidase–chlorite dismutase superfamily and the (4) peroxidase–peroxygenase superfamily.

Immunity against blood-borne pathogens: Several reports on mosquito immunity suggested the role of heme peroxidase in immunity against blood-borne antigens. There are several classes of peroxidases in *Anopheles* such as glutathione peroxidases, NADPH-dependent peroxidases, and heme peroxidases. Heme peroxidases have been found to associate with many immune responses directly or indirectly. The heme peroxidase HPX15 with dual oxidase forms a mucin barrier along the lumen side of midgut post blood feeding and creates a low immunity zone (Kumar et al., 2010). *Plasmodium* takes advantage of this low immunity zone and hides in the midgut. Another report in *An. gambiae* showed that heme peroxidase HPX2/NOX5 (NADPH oxidase) system has antiplasmodial response during *Plasmodium* development in the midgut. This system potentiates the toxicity of NO (nitric oxide) and enhances the clearance of *Plasmodium* via TEP1 mediated lysis (de Almeida Oliveira et al., 2012). So, mucin barrier formed by HPX15/Duox system protects *Plasmodium* from immune responses while at the time of midgut traversal, the midgut epithelial HPX2/NOX5 system enhanced their killing. In this way, heme peroxidases are reported to modulate the mosquito immunity.

Immunity in mosquito hemolymph: Upon microbe infection, cell adhesion is critical in the cellular immune response in invertebrates (Johansson, 1999). Peroxinectins are reported as cell adhesion molecules having a peroxidase domain and an integrin binding motif. They function in degranulation, encapsulation enhancement, opsonization, and peroxidase (Johansson and Söderhäll, 1989); (Kobayashi et al., 1990); (Thörnqvist et al., 1994; Johansson et al., 1995). The activity of these molecules

is generated with concomitant activation of the prophenoloxidase (proPO) system (Johansson et al., 1995). When a foreign particle enters the hemolymph, hemocytes recognize the foreign intruder as non-self and change from non-adhesive to adhesive cells, strongly adhering to the foreign target. The semi-granular and granular cells attach and spread across the surface of the foreign intruder, and subsequently form a multilayered sheath of cells during encapsulation.

In freshwater crayfish *Pacifastacus leniusculus*, peroxinectin is synthesized and stored in granulocytes in an inactive form, is released during the degranulation response, and activated outside the cells to mediate hemocyte attachment and spreading (Johansson and Söderhäll, 1988; Liang et al., 1992). Peroxinectin has been isolated and purified from crayfish *P. leniusculus*, *Litopenaeus vannamei* and tiger shrimp *Penaeus monodon*, and demonstrated to have cell adhesion and peroxidase activities in the presence of lipopolysaccharide (LPS) and β-1,3-glucans (Johansson and Söderhäll, 1988; Sritunyalucksana et al., 2001; Liu et al., 2004).

Moreover, extracellular SOD (EC-SOD) and integrin (a peripheral membrane protein) form a complex with peroxinectin and presumably, localize at the cell surface. The binding of the cell surface SOD to the cell-adhesive/opsonic peroxinectin may mediate, or regulate, cell adhesion and phagocytosis; it may also be important for the efficient localized production of microbicidal substances (Johansson et al., 1999). Hence, studying the function of heme peroxidases in mosquito immunity may explore their potential role in limiting the *Plasmodium* development.

1.8 Heme peroxidases play a crucial role in insect development

Heme peroxidases have been reported to also play crucial role in both development as well as propagation of insects. One heme peroxidase HPX15 is reported to function as female spermathecal detoxifying enzyme in *An. gambiae* (Shaw et al., 2014). Another HPX, *Cysu* is crucial for wing maturation process in *Drosophila*. (Bailey et al., 2017). HPXs are also found in eggs of several insects where they function in mosquito oogenesis as well as eggshell (or chorion) hardening. Eggshell typically consists of two layers- inner vitelline membrane and outer chorion layer (each of the layers are made of several proteins that are hardened by di- and tri- tyrosyl crosslinks among the proteins) (Amenya et al., 2010).While *An. gambiae* HPX- AgHPX8 is reported in oogenesis, DUOX and a peroxinectin-like molecule has been widely reported to catalyse chorion protein crosslinking (chorion hardening is crucial for eggs of insects as it prevents them from desiccation) (Amenya et al., 2010; Li et al., 1996; Li and Li, 2006).

Gaps in Existing Research

The knowledge of innate immunity in mosquitoes from Indian subcontinent is least explored. The role of mosquito cellular immunity against very early stages of *Plasmodium* infection is not well reported. Also, the role of midgut bacteria in the immunity of *An. stephensi* is not very well elucidated. Hence, the understanding of immune responses in *An. stephensi* is a prerequisite to developing malaria transmission blocking strategies. Heme peroxidases play a crucial role in mosquito innate immune responses, and thus elucidation of their role in the regulation of pathogens can help in the selection of target molecules for further transmission blocking strategies.

Research Objectives

The objectives of this research work were focused to identify and characterize heme peroxidases (DBLOX and HPX8) from *An. stephensi* and to understand their role in the regulation of mosquito innate immunity.

The proposed research objectives are:

Objective 1: The identification of blood-induced and immune-regulatory peroxidases(s) in the mosquito gut/carcass.

Objective 2: Analysis of peroxidases gene structure, regulatory elements and expression kinetics.

Objective 3: To study the effect of peroxidase(s) silencing on the crosstalk between different immune pathways.

Chapter 2

Materials and Methods

2.1 Rearing of mosquitoes

Anopheles stephensi mosquitoes were reared in an insectary maintained at 28^{o}C, 80% relative humidity and 12h light: dark cycle. Adult mosquitoes were maintained on 10% sucrose solution soaked in cotton balls. After five days of eclosion, starved females were fed on anesthetized Swiss albino mice. Gravid females were allowed to lay eggs on moist Whatman paper. Hatched larvae were fed on a 1:1 mixture of dog food (PetLover's crunch milk biscuit, India) and fish food (Gold Tokyo, India) as described before (Kajla et al., 2016b). The mice for the experimental purpose were maintained in Central Animal Facility, BITS Pilani and all the procedures were approved by the Institutional Animal Ethics Committee (Protocol No: IAEC/RES/18/02).

2.2 Retrieval of heme peroxidase genes from *An. stephensi* genome

Heme peroxidases (HPX) – DBLOX and HPX8, were previously reported as important molecules of midgut immunity against blood-borne antigens in *An. gambiae* (Kumar et al., 2004; Kumar et al., 2010a). Therefore, we attempted to characterize them from the Indian malaria vector *An. stephensi*. Briefly, their sequences (listed in **Table 2.1**) were retrieved from *An. gambiae* and individually blasted against *An. stephensi* genome (taxid: 30069) available at NCBI to retrieve putative contig that contained the ortholog of respective AgHPX gene. Contigs listed as best hit of BLAST result were further considered to recover the putative heme peroxidase gene from *An. stephensi* genome. The obtained contigs were further analyzed using GENSCAN web server (Burge and Karlin, 1997) to predict the HPX gene. The putative *An. gambiae* HPX gene ortholog in *An. stephensi* genome along with their respective contig, SuperContig, and Ensembl identifier are listed in **Table 2.1**.

Table 2.1: List of heme peroxidase genes. GeneID of *An. gambiae* heme peroxidases (DBLOX. HPX8) and their respective orthologs in *An. stephensi* along with their contig number, supercontig, and Ensemble identifier are listed in the table.

S. No.	*An. gambiae* Heme peroxidase (Gene ID)	Ortholog in *An. stephensi* (nucleotide location)	Genomic location in *An. stephensi* genome		
			Contig number	Supercontig	Ensembl identifier
1	AgDBLOX (AGAP008350)	AsDBLOX (1,904,022-1,909,430)	1690	KB664621	ASTE008679
2	AgHPX8 (AGAP004038)	AsHPX8 (542,922- 545,373)	4435	KB664356	ASTE008825

2.3 Designing of *An. stephensi* heme peroxidase gene-specific primers

From the putative *An. stephensi* contigs obtained above, full-length heme peroxidase genes were predicted using GENSCAN web server (Burge and Karlin, 1997). The nucleotide sequences of the predicted gene and the respective AgHPX ortholog were aligned to design gene-specific primers (5′ to 3′). **Table 2.2** lists the various primers used in this study.

Table 2.2: List of primers used in the study. The primers used to amplify and sequence the respective *An. stephensi* gene fragment along with primers for other genes are provided below.

Primer Set	Primer sequence (5′-3′)	Template (PCR product bp)		Purpose	References
		cDNA	gDNA		
AsDBLOX F1 AsDBLOX R1	CCACGTATCCTCAGCTGGAAC GGCCAAGGTCTTCCCAGGT	482	482	Sequencing	Present study
AsDBLOX F2 AsDBLOX R2	ATCTCTTCCCGGGCGGTAT GCCAGCTGGGCACAGTCGG	466	712	Real-time PCR	Present study
AsDBLOX F3 AsDBLOX R3	AGATAGAATTCCAAGAATG GATGGGTTACAGGTTGGGTTG	2951	3529	Sequencing	Present study
AsDBLOX F4 AsDBLOX R4	CTTATCTGAACGCACGGCT CGGAACGCGTCGGATGCCCC	2630	2966	Sequencing	Present study
AsDBLOX F5 AsDBLOX R5	CGATGCTTCCACCGGACATGT A CCGAACACCGTGCCCAAGGC G	360	360	Cloning, dsRNA preparation	Present study
AsHPX8 F1 AsHPX8 R1	AAGCTGGCAAACATCGACC GAACGGATAGCCGGCCGCAC C	342	342	PCR	Present study
AsHPX8 F2 AsHPX8 R2	CCTTAACCCGTCCCGTCTGC GTGTCGCTCGCCTCAGTCAT	509	509	Cloning, dsRNA preparation	Present study
AsHPX8 F3 AsHPX8 R3	GATCCTTTGCCGATGCGCTCA AT GTGCGACATAAACTGCCCGAA CTG	381	381	Real-time PCR	(Kajla et al., 2016a)
AsHPX8 F8 AsHPX8 R7	GTTGAAGTCAGTGCATCGACC GC CTCATACTGCAAAAGAGTACA TAAAACA	2543	2683	Sequencing	Present study
AsDUOX F4 AsDUOX R4	GCGTTCCTGGACAAGGAGAT GGCGTCACGAATTCTGGTAA	624	624	Real time PCR	Present study
AsHPX7 F1 AsHPX7 R1	GATCTGCACCATCCGCAG ATGTTGATGGCCGCCAGA	445	445	Real-time PCR	Present study

Primer Set	Primer sequence (5′-3′)	Template (PCR product bp)		Purpose	References
		cDNA	gDNA		
NOS F NOS R	ACATCAAGACGGAAATGGTTG ACAGACGTAGATGTGGGCCTT	250	382	Real-time PCR	(Luckhart et al., 1998)
GNBP F GNBP R	GAGTTCCAGTGGTACACCAACA CTTCGGCAGCAACCAGAT	333	493	Real-time PCR	(Kajla et al., 2016a)
EC-SOD F EC-SOD R	GGCGATCTTGGCAACATAAT TCGGCAAAGTATTCTTCCCG	296	296	Real time PCR	Present study
TEP1 F TEP1 R	GCTATCAAAATCAGATGCGCTATC ATCACAACCGCATGCTTCA	325	325	Real time PCR	(Kajla et al., 2016a)
16S rRNA F 16S rRNA R (universal primers for bacteria)	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTAATCCTG TT	467	467	Real time PCR	(Kumar et al., 2010b)
Pb 28S rRNA F Pb 28S rRNA R	CGTGGCCTATCGATCCTTTA GCGTCCCAATGATAGGAAGA	168	168	Real Time PCR	(Brandt et al., 2008)
S7 F S7 R (housekeeping gene)	GGTGTTCGGTTCCAAGGTGA GGTGGTCTGCTGGTTCTTATCC	487	600	Internal loading controls	(Vijay et al., 2011)
M13 F T7-M13 R	GTAAAACGACGGCCAGT CTCGAGTAATACGACTCACTATAG GGCAGGAAACAGCTATGAC	Variable (depending on the construct)	For dsRNA preparation	dsRNA preparation	pCR-II TOPO TA-Vector® kit

2.4 PCR amplification, cloning and full-length sequencing of heme peroxidase genes

The above-mentioned gene-specific primers, as mentioned in **Table 2.2**, were used to PCR amplify either the partial or full-length HPX gene from the cDNA template. The PCR products were sequenced to confirm the presence of HPX gene in the retrieved contig of *An. stephensi*. These fragments were further cloned to prepare dsRNA for gene silencing approach.

To amplify fragment sizes of less than 1kb, Thermo Scientific Taq polymerase was used. While for longer fragments Phusion High-Fidelity DNA Polymerase (Thermo scientific, #F-530S) was used. cDNA of sugar-fed female carcasses was used as a template to amplify full-length *An. stephensi* DBLOX, i.e. AsDBLOX gene, while full-length *An. stephensi* HPX8, i.e. AsHPX8 gene was amplified using cDNA of 24 h blood-fed female midguts as a template; each gene was amplified using primers mentioned in

Table 2.2. The PCR using Phusion High-Fidelity DNA Polymerase was initiated at 98^{o}C for 30 s and then followed by 35 cycles at 98^{o}C for 10 s, 62^{o}C for 30 s and 72^{o}C for 2 min. The final extension was carried out at 72^{o}C for 10 min. The PCR using Thermo Scientific Taq polymerase was initiated at 95^{o}C for 30 s and then followed by 35 cycles at 95^{o}C for 20 s, 55-58^{o}C for 50 s and 72^{o}C for 2 min. The final extension was carried out at 72^{o}C for 10 min. The PCR product was purified and sequenced commercially at Eurofins genomics (www.eurofinsgenomics.eu). The sequence identity of full-length AsDBLOX and AsHPX8 genes were confirmed through BLAST results and their sequences were submitted to GenBank NCBI. The GenBank accession numbers for *An. stephensi* AsDBLOX and AsHPX8 are MF964191 and KY363391, respectively.

2.5 Malaria parasite *Plasmodium berghei* maintenance and mosquito infection

P. berghei (ANKA strain) was provided by Dr. Asif Mohammad, Scientist, ICGEB, New Delhi, India), and a transgenic *P. berghei* PbGFP, expressing GFP in all developmental stages (Franke-Fayard et al., 2004) was a gift from Dr. Agam Prasad Singh, National Institute of Immunology, New Delhi, India. Both the *Plasmodium* strains were maintained in Swiss albino mice following the protocols as described before (Dong et al., 2009; Dhawan et al., 2015). The parasitemia of the infected mice was determined from blood films stained with 1% Giemsa under a light microscope. For blood stage passages, 100-150 µl of blood from an infected mouse (parasitemia ~20%) was intraperitoneally injected into a healthy mouse. Parasitemia in mouse blood was determined, and potential infectivity to mosquitoes was established using exflagellation assays as described before (Billker et al. 1997; Kajla et al., 2017). Briefly, 2 µl of tail blood was immediately mixed with 10 µl of exflagellation buffer (10mM Tris-Cl pH 8, 150mM NaCl and 10mM Glucose) and 10ul Fetal bovine serum (FBS), on a microscope slide and covered with cover-slip. The slide was observed under a microscope. In all the experiments, mice having ~5-7% parasitemia with 2-3 exflagellations per field observed under 40X objective were used for mosquito infection.

Four to five days old and overnight starved female mosquitoes were fed on anesthetized Swiss albino mouse infected with the wild-type or GFP expressing *P. berghei.* After feeding the females were maintained at 21°C, which is a permissive temperature for *P. berghei* development (Kumar et al., 2010b; Dhawan et al., 2015). Female mosquitoes were starved for ~12 h before feeding to ensure engorgement. Uninfected blood fed (BF) or *P. berghei* infected blood (Pb) fed midguts were dissected

from a pool of mosquitoes (n=20) at different time points of feeding. The dissected midguts or carcasses (rest of the body except midgut) were kept in RNAlater™ (Qiagen) and stored at -80°C. Sugar-fed (SF) midguts or carcasses served as controls and were also collected in a similar way.

2.6 Mosquito tissue collection

2.6.1 Tissue collection from different developmental stages and body compartments of *An. stephensi*.

Different developmental stages of *An. stephensi,* i.e. eggs, larvae (first to fourth instar), pupae, adult males, and females were collected separately in RNAlater™ (Qiagen) and stored at -80°C. In some experiments, *An. stephensi* females were allowed to feed on an anesthetized mouse and Midguts (Mg) and Carcasses i.e. rest of the body (Cc), were collected and stored as discussed before.

2.6.2 Hemocyte collection from *An. stephensi*.

In some experiments, hemocytes were collected from adult female mosquitoes. For that, 207nl solution of Ashburner's Phosphate Buffer Saline (PBS) (3mM Sodium chloride, 7mM disodium hydrogen phosphate, and 3mM sodium dihydrogen phosphate pH 7.2) supplemented with FBS (Fetal Bovine Serum) in a ratio of 1:2 was injected into the thorax of each cold anaesthetized mosquitoes using Nanoject II microinjection system (Drummond). After 5 min, extreme tip of the each mosquito abdomen was pricked with a sterile scalpel, and the thorax was further flushed with 2µl FBS-PBS solution. This caused flushing of mosquito hemocytes out of the pricked abdomen. The flushed solution was immediately collected in Eppendorf tubes containing 10µl RNAlater™. The midguts and carcasses were collected from the same mosquitoes post flushing of the hemocytes and samples were stored at -80°C.

2.6.3 Bacterial challenge to *An. stephensi* IVth instar larvae or adult females

Escherichia coli (Gram-negative bacterium, MTCC 40) and *Micrococcus luteus* (Gram-positive bacterium, MTCC 106) were procured from MTCC (Microbial Type Culture Collection), Institute of Microbial Technology, Chandigarh, India. Bacterial cultures were grown to an optical density of A_{600}=0.5 in LB (Luria-Bertani) broth, and 500 µl of each culture was mixed and centrifuged for 5 min at 2300*xg*. The resulting pellet was washed twice with Ashburner's PBS. The cells pellet were resuspended in 125 µl Ashburner's

PBS and 69 nanoliters (~69000 bacteria/larvae) were injected into the thorax of 4th instar *An. stephensi* larvae using a nano injection system (Nanoject II microinjection system; Drummond).

In some experiments adult females were also inoculated with 69 nanoliters of bacteria (~69000 bacteria/mosquito) by the similar process mentioned above and midguts, carcasses and hemolymph (containing hemocytes) were collected at different time points of injection by the process described above in **Section 2.6.2** and stored at -80°C. In some experiments, one-day-old female mosquitoes were injected with dsRNA. After four days, they were injected with bacteria and their midguts, carcasses and hemocytes were also collected as above and stored at -80°C.

2.6.4 *An. stephensi* bacterial feeding and tissue collection

The two bacterial strains - *E. coli* and *M. luteus* were individually grown in LB media and pelleted in the same manner as described in section 2.6.2. The pellet was resuspended either in saline or mouse blood to make the final count of 10^9cells/ml (finally 0.5x10^9 cells of each bacterium per ml). *An. stephensi* adult female mosquitoes were allowed to feed on saline alone (control), or bacteria supplemented saline with 10^9 bacterial cells/ml through an artificial membrane feeder as described before (Gupta et al., 2009). Mosquitoes fed on sugar or blood alone served as controls and was also collected similarly.

2.7 dsRNA synthesis

A 218-bp fragment of the lacZ gene (control) was amplified using the primers (5' to 3') F-GAGTCAGTGAGCGAGGAAGC and R-TATCCGCTCACAATTCCACA and cloned into the pCRII-TOPO vector (Gupta et al., 2010). The gene-specific primers used to amplify the segment of HPX gene for dsRNA synthesis are- AsDBLOX F5-R5 (360 bp) and AsHPX8 F2-R2

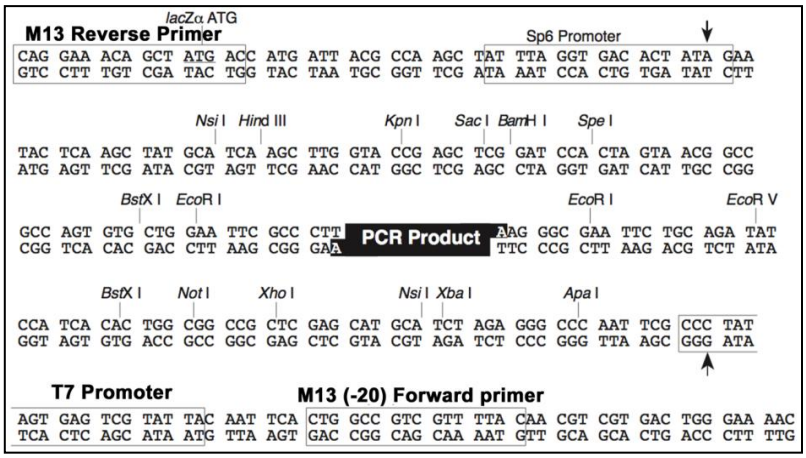


Figure 2.1: The pCRII-TOPO-TA vector map. The vector map demonstrating the position of the PCR product insert, position of T7 promoter, and location of M13F and M13R primers (https: //tools.thermofisher.com/content/sfs/manuals/topota_man.pdf)

(509 bp). The PCR product was purified and processed to add 3′ A tail and inserted into pCR-II TOPO TA-Vector® (Invitrogen Cat No K46001-01) following the manufacturer's instructions. The recombinant plasmid was used to transform high efficiency DH5-α TOPO10 *E. coli* chemically competent cells provided with pCR-II TOPO TA-Vector® kit. Few white colonies (Blue/white screening) were screened through colony PCR with M13 universal primers (**Figure 2.1)**. The recombinant colony that had the insert was used for plasmid isolation. This recombinant plasmid already has a T7 promoter site at the M13F end as shown in **Figure 2.1**.

However, T7 promoter at the other end of the fragment was incorporated by amplifying the inserted fragment using the following primers: M13F and T7-M13R (**Table 2.2**). The PCR reaction was performed as follows: an initial step of 5 min at 94°C, followed by 40 cycles with denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. After the final cycle, the product was extended for 10 min at 72°C. Amplicons were electrophoresed and extracted from the gel with the QIAquick Gel Extraction Kit (cat no 28704, Qiagen, Valencia, CA, USA). Gel purified PCR product was used to synthesize dsRNAs (*In vitro* transcription) using the MEGAscript kit (Cat No. AM1626, Ambion, Austin). dsRNA was then purified with the help of Microcon YM-100 filter (Millipore) and finally concentrated to 3 µg/µl in DNase- and RNase-free water.

2.8 Analyzing the effect of AsDBLOX gene silencing on bacteria

One to two-day-old female mosquitoes were injected with 69 nanoliters of 3 µg/µl (207 ng/mosquito) dsAsDBLOX into their thorax using a nanojector (Drummond, Broomall, PA, USA). Control mosquitoes were injected with dsLacZ in the same manner. Four days after the dsRNA injection mosquitoes were injected with 69 nanoliters mixture of *E. coli* and *M. luteus* bacteria ($0.5x10^9$ cells of each bacterium per ml of saline) and their midguts, carcasses or hemolymph (containing hemocytes) were collected at different time points and stored at -80°C following the methods outlined in **Section 2.6.4**. The efficiency of gene silencing, as well as its effect on bacterial growth, was calculated against dsLacZ injected bacteria challenged mosquitoes as controls using semiquantitative PCR.

2.9 Analyzing the effect of AsHPX8 gene silencing on AsHPX7 gene

One to two-day-old female mosquitoes were injected with 69 nanoliters of 3 µg/µl (212 ng/mosquito) dsAsHPX8 into their thorax using a nanojector (Drummond, Broomall, PA, USA). Control mosquitoes were injected with dsLacZ in the same manner. Four days

after the injection, mosquitoes were fed on anesthesized mice blood and their midguts and carcasses were collected at different time points and stored at -80°C following the methods outlined in **Section 2.6.4**. The efficiency of gene silencing was calculated against dsLacZ injected anesthesized mice blood fed mosquitoes as control using semiquantitative PCR.

2.10 RNA isolation and cDNA preparation

Total RNA was isolated from the above-mentioned tissue samples using an RNAeasy mini kit from Qiagen (Cat no. 74104) with slight modification, 30 µl β-mercaptoethanol (2-ME) per 1 ml RLT buffer. First-strand cDNA was synthesized using Quantitect reverse transcription kit (Qiagen Cat no. 205311) following manufacturer's instructions.

2.11 Expression analysis of various genes in different tissue samples using real-time PCR

The mRNA expression analyses of heme peroxidases and other immune genes were carried out through real-time PCR using SYBRgreen supermix in an IQ5 multicolor real-time PCR detection system (Bio-Rad), where ribosomal protein subunit S7 mRNA was used as internal loading control for normalization as described before (Kumar et al., 2010b; Salazar et al., 1993; Kajla et al., 2016b). The primers set used for heme peroxidase, and other immune genes for qPCR are mentioned in **Table 2.2**. PCR cycle parameters were as follows: initial denaturation at 95°C for 5 min, 40 cycles of 20 s at 94°C, 30 s at 57°C, and 50 s at 72°C. Fluorescence readings were taken at 72°C after each cycle. For final extension, incubation at 72°C for 10 min was completed and then subjected to a melting curve analysis, to confirm the identity of PCR product. Fold values of mRNA expression were calculated against respective controls using the $^{\Delta\Delta}$Ct method as described before (Livak and Schmittgen, 2001).

2.12 Analysis of conserved domains in AsDBLOX and AsHPX8 protein sequences

Conserved domains in full-length AsDBLOX and AsHPX8 genes were identified using the Conserved Domain Database (CDD) search tool available online at NCBI (Marchler-Bauer et al., 2014) and SMART database (Letunic and Bork, 2017). The results obtained from these analyses were further compared with the respective ortholog of *An. gambiae* to identify the conserved domains present in the HPX genes of both the species.

2.13 Selection of heme peroxidases for phylogenetic analysis

For analyzing the evolutionary relationship, we performed PSI-BLAST (Position-Specific Iterative Basic Local Alignment Search Tool) of full-length AsDBLOX and AsHPX8 proteins, and the resulting HPX protein sequences of various organisms were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/) and VectorBase database (https://www.vectorbase.org) (listed in **Table 2.3** and **Table 2.4**). It derives a position-specific scoring matrix (PSSM) or profile from the multiple sequence alignment of sequences detected above a given score threshold using protein-protein BLAST. Thus, PSI-BLAST provides a means of detecting distant relationships between proteins. In addition, *Arabidopsis thaliana* heme peroxidases were also included to understand the divergence of AsHPX from plants. The phylogenetic tree was constructed using the full-length protein sequences of all the peroxidases including AsDBLOX and AsHPX8.

Phylogenetic trees for AsDBLOX and AsHPX8 genes were constructed separately, from selected peroxidases (**Table 2.3 and 2.4**) using the maximum likelihood (ML) method implemented in MEGA 7.0 program. We aligned all protein sequences by Clustal W algorithm in the MEGA 7.0 program as before. The following criteria were selected for the phylogenetic analysis: 'WAG' option was selected as the model for amino acid substitution as this model best fits our data. For gaps and missing data, we used 'all sites' option. The ML tree was generated using nearest neighbor interchange (NNI) tree search algorithm. Branching pattern reliability was tested for both ML, by 1000 bootstrap replicates. The resulting phylogenetic tree was analyzed based on clusters and nodes formed. Because the topologies of phylogenetic tree obtained by ML and NJ methods were similar, therefore, only the ML tree for respective analyses is presented in this study.

Table 2.3: Sequences used for the phylogenetic analysis of AsDBLOX protein. The sequences selected for the analysis with their respective Phylum (Taxonomical hierarchy), vectorial capacity, Genus and species (abbreviation), Genbank or ensemble identifier and query coverage and percentage identity have been mentioned. * MF964191 is the full length AsDBLOX protein sequence.

Sl No.	Description	Organism (abbreviation)	Ensembl identifier or gene bank identity	% query coverage	% Identity
Phylum Arthropoda					
Subphylum: Hexapoda					
Class Insecta					

01	Order: Diptera	*Anopheles gambiae (Ag)*	XP_317106.5	95	96
02		*Anopheles sinensis (As)*	KFB45586.1	97	92
03		*Aedes aegypti (Aae)*	XP_001648059.2	96	83
04		*Aedes albopictus (Aal)*	KXJ70361.1	96	83
05		*Culex quinquefasciatus (Cq)*	XP_001862036	96	82
06		*Anopheles darlingi (Ad)*	ETN67505	87	89
07		*Musca domestica (Md)*	XP_005179733.1	95	64
08		*Stomoxys calcitrans (Sc)*	XP_013100427.1	96	63
09		*Bactrocera dorsalis (Bd)*	XP_011210093.1	96	62
10		*Ceratitis capitata (Cca)*	XP_004531322.1	96	62
11		*Lucilia cuprina (Lc)*	KNC29858.1	96	63
12		*Drosophila melanogaster (Dm)*	NP_609883.1	93	62
13		*Rhagoletis zephyria (Rz)*	XP_017485610.1	94	63
14	Order: Lepidoptera	*Papilio xuthus (Px)*	KP193000	95	59
15		*Bombyx mori (Bm)*	XP_012551293.1	95	60
17		*Amyelois transitella (Atr)*	XP_013198668.1	92	59
18	Order: Coleoptera	*Nicrophorus vespilloides (Nv)*	XP_017785026.1	95	59
19		*Agrilus planipennis (Ap)*	XP_018331432.1	93	58
20		*Anoplophora glabripennis (Agl)*	XP_018561865.1	93	58
21		*Oryctes borbonicus*	KRT83256	92	62
22	Order: Hymenoptera	*Polistes dominula (Pd)*	XP_015184442.1	95	57
23		*Neodiprion lecontei (Nl)*	XP_015511362.1	95	57
24		*Bombus impatiens (Bi)*	XP_012238752.1	95	58
25		*Megachile rotundata (Mr)*	XP_003705576.1	95	58
26		*Harpegnathos saltator (Hs)*	XP_011150582.1	95	57
27		*Linepithema humile (Lh)*	XP_012217334.1	96	56
28		*Cephus cinctus (Cci)*	XP_015597950.1	95	58
29		*Nasonnia vitripennis*	XP_016839299	74	41
30		*Ceratina calcarata (Ccal)*	XP_017887733.1	95	58
31		*Monomorium pharaonis (Mp)*	XP_012522560.1	95	57
32		*Apis mellifera (Am)*	XP_006558124.1	93	57
33		*Orussus abietinus (Oa)*	XP_012284279.1	95	57
34		*Dinoponera quadriceps (Dq)*	XP_014470094.1	95	56
35		*Eufriesea mexicana (Em)*	XP_017763304.1	95	57
36		*Wasmannia auropunctata (Wa)*	XP_011700902.1	96	55

37		*Camponotus floridanus (Cf)*	XP_011263566.1	95	55
38		*Vollenhovia emeryi (Ve)*	XP_011873477.1	96	56
39		*Dufourea novaeangliae (Dn)*	KZC13921.1	97	56
40		*Solenopsis Invicta (Si)*	XP_011156220.1	95	56
41		*Trachymyrmex cornetzi (Tc)*	KYN14715.1	95	56
42		*Cerapachys biroi (Cb)*	XP_011335795.1	95	56
43		*Atta colombica (Ac)*	KYM82275.1	95	56
44		*Acromyrmex echinatior (Ae)*	XP_011052619.1	92	57
45		*Athalia rosae (Ar)*	XP_012256198.1	93	59
46		*Cyphomyrmex costatus (Cco)*	XP_018403790	94	56
47		*Habropoda laboriosa (Hl)*	XP_017790311	95	55
48		*Trichogramma pretiosum (Tp)*	XP_014237867	94	56
49		*Pseudomyrmex gracilis*	XP_020291060	95	57
50		*Fopius arisanus*	XP_011301386	94	56
51		*Pogonomyrmex barbatus*	XP_011641998	93	57
52		*Copidosoma floridanum*	XP_014214058	93	56
53		*Ceratosolen solmsi marchali*	XP_011498272	93	56
54		*Diachasma alloeum*	XP_015127618	93	59
55		*Melipona quadrifasciata*	KOX68884	94	65
56	Order: Hemiptera	*Helicoverpa armigera*	XP_021190953	95	59
57		*Halyomorpha halys*	XP_014285769	93	54
58		*Lygus hesperus*	JAQ17951	92	50
59		*Bemisia tabaci*	XP_018902400	90	50
60		*Diaphorina citri*	XP_008472721	82	62
61		*Danaus plexipus plexupus*	OWR55548	67	64
62		*Cuerna arida*	JAS57026	89	56
63		*Diuraphis noxia*	XP_015373617	74	42
64		*Clastoptera arizonana*	JAS30120	74	41
65	Order: Phthiraptera	*Pediculus humanus corporis*	XP_002425566	93	50
66	Order: Isoptera	*Zootermopsis nevadensis*	XP_021931926	92	46
Subphylum: Crustacea					
67		*Daphnia pulex*	EFX88723	90	46
68		*Hyalella azteca*	XP_018012596	91	44
Phylum Nematoda					
69	Class: Secernentea	*Ancylostoma ceylanicum*	EYC08356	90	36
70		*Toxocara canis*	KHN82375	90	34
71		*Haemonchus contortus*	CDJ81898	90	35
72	Class: Adenophorea	*Trichinella pseudospiralis*	KRZ80717	91	35
73	Class: Chromadorea	*Caenorhabditis elegans*	NP_496407	90	36

74	*Arabidopsis*	Peroxidase	AAA32849.1	Outgroup

Table 2.4: Selected heme peroxidases for phylogenetic analysis of AsHPX8 protein.

Sl. No	Organism (abbreviation)	Accession No.	% Query coverage	% identity
Phylum Arthropoda				
01	*An. gambiae* (Ag)	XP_309592	92	78
02	*An. sinensis* (Asi)	KFB41290	92	69
03	*An. darlingi* (Ad)	ETN62073	94	65
04	*An. aquasalis* (Aaq)	JAA98995	79	60
05	*Culex tarsalis* (Ct)	JAV19172	89	52
06	*Culex quinquefasciatus* (Cq)	XP_001849234	89	52
07	*Aedes albopictus* (Aal)	XP_019526999	91	48
08	*Aedes aegypti* (Aae)	XP_001649030 XP_001649029	91	49
09	*Corethrella appendiculata* (Cap)	JAB57279	89	42
10	*Lucilia cuprina* (Lcu)	KNC22326	89	43
11	*Bactrocera dorsalis* (Bd)	JAC48832	76	47
12	*Stomoxys calcitrans* (Sca)	XP_013101016	74	46
13	*Musca domestica* (Md)	XP_011292728	89	42
14	*Drosophila melanogaster* (Dm)	NP_650648	91	41
15	*Ceratitis capitata* (Cca)	XP_004529225	89	41
16	*Ragholetis zephyria* (Rz)	XP_017471463	70	49
17	*Clunio marinus* (Cma)	CRK86945	89	39
18	*Dendroctonus ponderosae* (Dpo)	XP_019754370	75	42
19	*Aethina tumida* (Atu)	XP_019868210	89	38
20	*Nyssomyia neivai* (Nn)	JAV13528	67	45
21	*Nicrophorus vespilloides* (Nv)	XP_017778752	60	47
22	*Agrilus planipennis* (Apl)	XP_018323744	88	38
23	*Trobolium castaneum* (Tca)	XP_008201340	94	38
24	*Anoplophora glabripennis* (Agl)	XP_018562405	67	42
25	*Papilio polytes* (Pp)	XP_013137630	94	35

26	*Bombyx mori* (Bm)	XP_012545081	89	36
27	*Amyelois transitella* (Atr)	XP_013192839	67	43
28	*Plutella xylostella* (Pxy)	XP_011557712	67	41
29	*Ixodes scapularis*(Is)	XP_002406316	79	39
30	*Orchesella cincta* (Oci)	ODM94915	70	39
31	*Hyalella azteca* (Haz)	XP_018024847	67	42
32	*Daphnia magna* (Dma)	JAN59725	94	33
33	*Macropthalmus japonicas* (Mj)	AID47197	86	37
34	*Arabidopsis*	AAA32849.1	Outgroup	
		CAB37193		

2.14 *In silico* analysis of AsDBLOX and AsHPX8 gene structure

The putative signal peptide sequence at the N-terminal of protein was predicted using SignalP software (Petersen et al., 2011). Protein 3D structure and secondary structure, were analyzed using RaptorX (Källberg et al., 2012) and Phyre^2 servers (Kelley and Sternberg, 2009), respectively. The generated models were further evaluated for quality and atomic content using different online servers like RAMPAGE (DePristo et al., 2003), ERRAT (Colovos and Yeates, 1993), PROCHECK (Laskowski et al., 1993) and VERIFY3D (Eisenberg et al., 1997). The obtained models were further refined by energy minimization using online server YASARA (Krieger et al., 2009). The molecular weight and isoelectric pH of heme peroxidase protein were analyzed online at http://web.expasy.org/compute_pi/ (Gasteiger et al., 2005). The nature of protein (cytoplasmic, non-cytoplasmic or presence of transmembrane domain) was analyzed using Phobius software {based on hidden Markov model (HMM)} (Käll et al., 2007).

2.15 Bioinformatics analysis of promoter, transcription factors, and regulatory elements of heme peroxidase genes

The 5′ upstream sequences of heme peroxidases genes were analyzed by **GENSCAN** (http://genes.mit.edu/GENSCAN.html) (Organism vertebrate and Suboptimal exon cutoff 1), (organism *D. melanogaster*) and **NNPP2.2** (http://www.fruitfly.org/seq_tools/promoter.html) (organism selected Eukaryotes and Minimum promoter score 0.8) servers to find the promoter of both the HPXs as mentioned before (Reese, 2001). The putative transcription binding sites in the 5′ upstream regulatory region of AsDBLOX

and AsHPX8 genes were determined by MatInspector (Cartharius et al., 2005) and JASPAR software (Mathelier et al., 2013). For these analyses, the transcription binding sites from insect family were selected along with other default parameters.

2.16 Analysis of sequence divergence of DBLOX between different taxonomic groups

DBLOX has two heme peroxidase domain, which likely evolved by gene duplication event. We analyzed Ka/Ks ratio (Synonymous / Nonsynonymous Substitutions) using tools available at http://services.cbu.uib.no/tools/kaks keeping the default parameter “none” which means that no codon bias was used in the algorithm. The window option was also set at none, i.e. no windows were used, and the whole sequence was averaged together. To analyze selective forces on each amino acid, we used Selecton server version 2.4 and Null model as an Evolutionary model with no positive selection (M8a, beta + w =1) as before (Stern et al., 2007; Doron-Faigenboim et al., 2005).

2.17 Analysis of duplicated paralog of AsHPX8

Vectorbase database reported *An. gambiae* HPX8 (AgHPX8) as paralog of AgHPX7. Hence, we retrieved the HPX8 and HPX7 genes from *An. gambiae* and based on a prediction by GENSCAN web server as well as Vectorbase; we predicted the respective orthologs in *An. stephensi*.

2.18 Statistical analysis of the data

All the data were expressed as a mean ± standard deviation. Statistical significance between test and respective controls was analyzed by Student’s t-test or one-way ANOVA post-Tukey's Multiple Comparison Test using GraphPad Prism 5.0 software (Motulsky et al., 1999). The data with $P \leq 0.05$ were considered significant. The sigma plot (SigmaPlot 10.0 Systat Software, San Jose, CA) and SigmaStat plus software (StatPlus v5, AnalystSoft Inc. statistical analysis program) were used to prepare graphs and performing the statistical analysis of the data, respectively.

Chapter 3

Identification and characterization of an *Anopheles* double peroxidase (AsDBLOX)

3.1 Abstract

In this chapter, we carried out the molecular characterization of a unique double heme peroxidase DBLOX from Indian malaria vector *An. stephensi*. This gene encodes for a 1402 amino acids long protein. The first domain lacks all the features of a complete functional heme peroxidase domain and instead has two integrin binding motifs- RGD (Arg – Gly – Asp) and LDV (Leu – Asp – Val). The second peroxidase domain, on the other hand, has all the features of a complete heme peroxidase domain. Thus the AsDBLOX gene is a peroxinectin. The DBLOX gene was compared between *An. gambiae* and *An. stephensi* AsDBLOX were compared and we observed that this gene is a true ortholog of AgDBLOX. Also, the gene is expressed in all the developmental stages of mosquito, with heightened expression during the pupal stage of life cycle. Pupa to adult molting involves a cascade of molecular changes and increased oxidative stress during stage coincides with the induction of AsDBLOX gene which suggested its possible role during mosquito metamorphosis.

3.2 Introduction

Heme peroxidases (HPXs) are the most abundant type of peroxidases and are present in all kingdoms of life (Zamocky et al., 2008). They catalyze H_2O_2-dependent oxidation of a wide variety of substrates and are involved in numerous processes like the innate immune responses, crosslinking of proteins within extracellular matrixes (ECM) as well as molecules within the cuticle and chorion of arthropods and nematodes (Soares et al., 2011; Konstandi et al., 2005; Bailey et al., 2017; Kajla et al., 2017b). Over 18 HPXs have been identified in African malaria vector - *An. gambiae* and several of them are reported to have regulatory effects against both rodent and human malaria parasites (Kumar et al., 2010; de Almeida Oliveira et al., 2012). However, the Afrotropical *An. gambiae* complex which includes closely related and morphologically indistinguishable mosquito species differs dramatically as major, minor and nonvectors based on their ability to transmit *Plasmodium*. They are reported to have diverged from the other worldwide distributed *Anopheles* due to environmental heterogeneities, notably those of anthropogenic origin (White et al., 2011). These differences in vector competence pointed to the necessity in elucidating the HPX mediated immune mechanism in *An. stephensi*.

3.3 Results

3.3.1 Cloning and characterization of AsDBLOX gene from *An. stephensi*

The reported AgDBLOX gene was blasted against *An. stephensi* genome (taxid: 30069) and its putative ortholog, now referred as AsDBLOX gene was identified in contig 1690 with SuperContig KB664621 and Ensembl identifier ASTE008679 in the partially annotated genome. The contig 1690 exhibited 99% query coverage between the nucleotide positions 20074 to 15434 (Supercontig KB664621 and Ensembl identifier ASTE008679). To confirm AsDBLOX gene in the genome of *An. stephensi,* the PCR-based approach was employed. Gene-specific primers F1, R1, F2, and R2, were designed based on nucleotide alignment between AgDBLOX and AsDBLOX as described in the **Materials and Methods**. *An. stephensi* cDNA (midgut or carcass) was used as a template to amplify AsDBLOX gene. The results presented in **Figure 3.1** showed the amplification of a single fragment of ~500 bp (expected size 484bp) from both the templates separately using F2R2 primers. Surprisingly, the F1R2 primers amplified a fragment of ~1000bp which was sequenced, and the final sequence

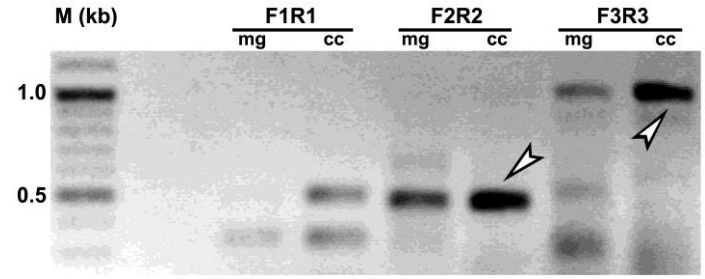


Figure 3.1: PCR amplification of *An. stephensi* AsDBLOX gene. *An. stephensi* midguts (mg) or carcass (cc) cDNA template was amplified using F1 and R2 primers and a single band of expected size ~1000 bp was observed in the gel, while a band of ~500bp was amplified by F2-R2. The left lane (M) represents the DNA ladder (SM03331-Thermo Fischer Scientific) used as reference for identifying the product size.

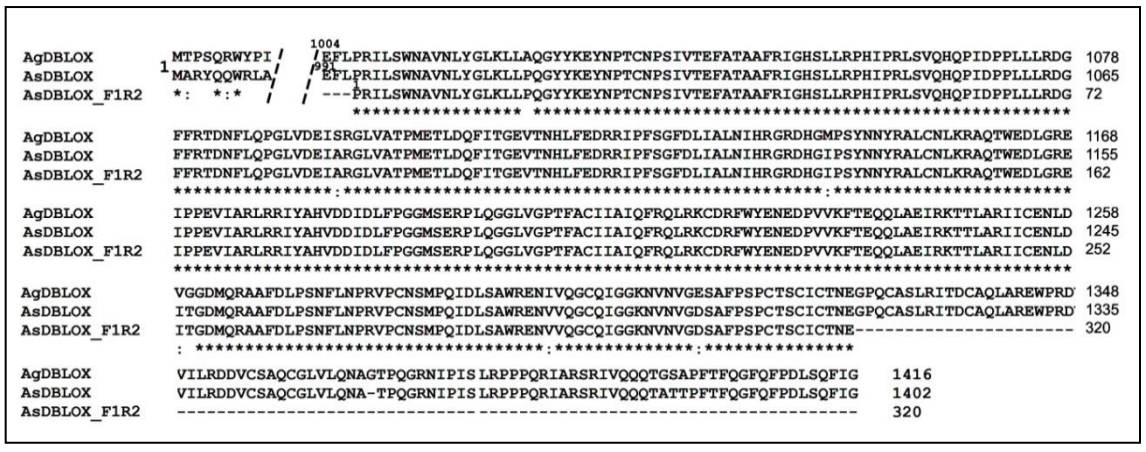


Figure 3.2: Amino acid alignment between AgDBLOX, AsDBLOX and the AsDBLOX F1R2 sequence. The reported AgDBLOX, predicted AsDBLOX and the obtained sequence from our lab i.e., F1R2 were aligned using Clustal Omega.

of 960bp was submitted to GenBank (MF616330). The nucleotide BLAST result of the sequence revealed its closest match with *An. gambiae* AgDBLOX (100% query coverage, 87% identity and E value 0.0). The amino acid alignment between AgDBLOX, AsDBLOX and the F1R2 sequenced portion of AsDBLOX is shown in **Figure 3.2**. This partial fragment confirmed the presence of DBLOX in *A. stephensi* genome.

3.3.2 AsDBLOX is an ortholog of AgDBLOX gene

The reported *An. gambiae* AgDBLOX gene (AGAP008350) did not start with the start codon ATG (**Figure 3.3A**). Then, we retrieved AgDBLOX genomic sequence with 1500bp from upstream as well as downstream region and analyzed with GENSCAN web

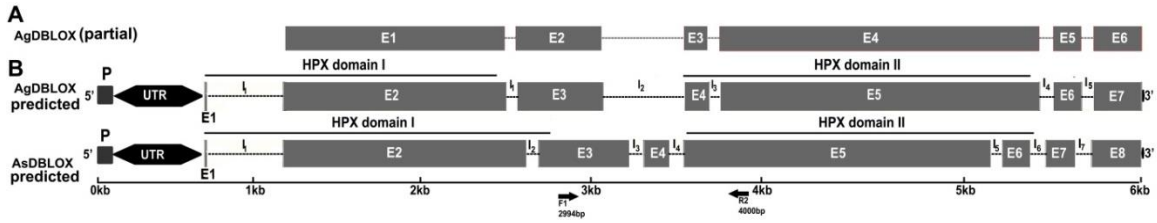


Figure 3.3: Genomic organization of AgDBLOX and AsDBLOX genes. A. AgDBLOX partial gene reported in NCBI. **B.** Predicted full length AgDBLOX has seven exons viz. E1 8 bp, E2 1323 bp, E3 491 bp, E4 136 bp, E5 1824 bp, E6 157 bp, E7 270 bp (total CDS predicted: 4209 bp) and six introns I_1 461 bp, I_2 65 bp, I_3 466 bp, I_4 67 bp, I_5 83 bp, I_6 73 bp respectively. The gene promoter (P) is of 40 bp. The 5´UTR and 3´UTR are of 261 bp and 34 bp, respectively. The predicted AsDBLOX gene has eight exons viz. E1 8 bp, E2 1326 bp and E3 491 bp, E4 136 bp, E5 1678 bp. E6 146 bp, E7 156 bp and E8 268 bp (total CDS 4206 bp) and seven introns I_1 422 bp, I_2 72 bp, I_3 84 bp, I_4 83 bp, I_5 69 bp, I_6 90 bp, I_7 94 bp respectively. The gene promoter (P) is of 40bp, and the 5´UTR) and 3´UTR are of 289 bp and 91 bp, respectively. Numbering indicates the nucleotide position of the orthologs. The positions of forward (F1) and reverse (R2) primers are depicted here (as mentioned in **Table 2.2**). The bioinformatics analyses for both the genes were performed using GENSCAN and NNPP2.2 web servers and MatInspector software.

server (Burge and Karlin, 1997), NNPP2.2 server and MatInspector software to predict full-length gene, its promoter and 5´or 3´UTRs as shown in **Figure 3.3B**. The full-length AgDBLOX was blasted against *A. stephensi* genome (taxid: 30069) and similar methodology as above was used to predict the full-length AsDBLOX gene (9.45 kb) using GENSCAN web server (**Figure 3.3B)**. The predicted AsDBLOX gene was found to be 5412 bp long with an open reading frame (ORF) of 4206 bp that encodes for a 1402 amino acids long protein. The predicted 5′-untranslated region (5′-UTR) and 3′-untranslated region (3′-UTR) were 289 bp and 91 bp, respectively. The signal for polyadenylation “TATAAA” was found 94 bp downstream from the stop codon in 3′-UTR. NCBI nucleotide BLAST of predicted AsDBLOX sequence revealed first hit as AgDBLOX with 95% query coverage, 87% identity and E value 0.0.

Table 3.1: **Different genomic organizations of DBLOX in *An. gambiae* and *An. stephensi***. The predicted AgDBLOX and AsDBLOX genes (TSS as +1) have seven and eight exons respectively, as analyzed by GENSCAN web server, NNPP2.2 server, Vectorbase and BLAST results.

Sl. No	Feature	AgDBLOX location		AsDBLOX location (TSS as +1)	
		Start	End	Start	End
01	Promoter	-40	-1	-40	-1
02	5′ UTR	+1	+261	+1	+289
03	Exon 1 (E1)	+262	+269	+290	+297
04	Exon 2 (E2)	+735	+2057	+720	+2045
05	Exon 3 (E3)	+2123	+2613	+2118	+2608
06	Exon 4 (E4)	+3080	+3215	+2693	+2828
07	Exon 5 (E5)	+3283	+5106	+2912	+4589
08	Exon 6 (E6)	+5190	+5346	+4659	+4804
09	Exon 7 (E7)	+5420	+5689	+4895	+5050
10	Exon 8 (E8)	Absent	Absent	+5145	+5412
11	3′ UTR	+5690	+5724	+5413	+5504
12	Poly A	+5725	+5730	+5506	+5512

A notable difference observed in **Figure 3.3** and described in **Table 3.1**, was the different exonic and intronic structures of AgDBLOX and AsDBLOX genes. This indicated the possible evolutionary pressure on this gene between the two *Anopheles* species. Thus, DBLOX has possibly evolved from a double domain architecture where the domains have originated by gene duplication to a possible state where either of the two results can occur - a. the HPX domain I may diverge further in the course of evolution by acquiring new mutations, hence acquiring a new function, or b. it may continue to retain the amino acid sequence in the HPX domain I and thus have some yet unknown role.

3.3.3 The Intronic structure of AsDBLOX is different from AgDBLOX

A noteworthy feature that we observed from analysis of genomic organization of both the genes was that an extra intron was present in *A. stephensi* while no such intron was observed in AgDBLOX (**Figure 3.3B**). This information prompted us to analyze the presence of the 5th intron in the genome of other anophelines. We found that the intron was absent only in the mosquitoes of *An. gambiae* complex – *An. coluzzi, An.*

arabiensis, An. merus, An. gambiae, An. quadriannulatus, An. melas, An. nili and *An. christyi* (**Figure 3.4**). The other *Anopheles* vectors have the 5th intron. This difference

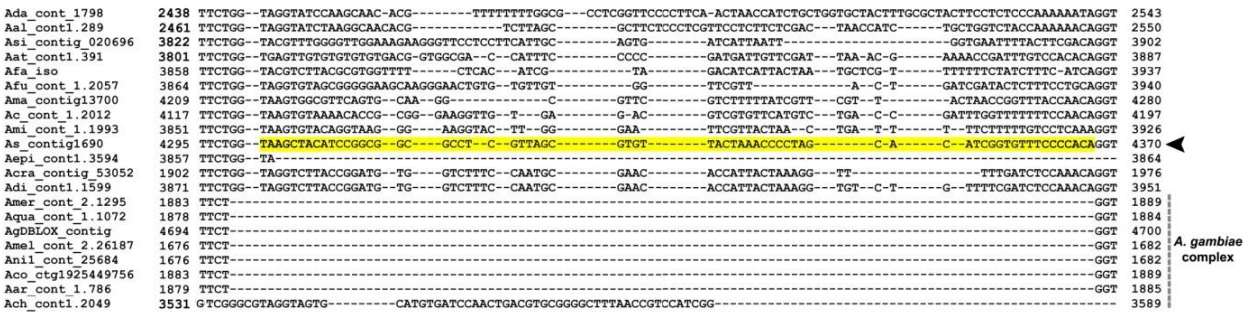


Figure 3.4: Intron 5 is absent in DBLOX of *An. gambiae* species complex. The genomic sequences of DBLOX *Anopheles* species obtained from BLAST results were aligned by Clustal Omega. The 5th intron is absent in the gambiae complex. The block arrow head is *An. stephensi* contig1690 and yellow highlighted region is the 5th intron.

indicated the divergence of DBLOX between the worldwide distributed *Anopheles* vectors and the African malaria vectors. However, *An. christyi,* a non-vector of *An. gambiae* complex, showed variation as compared to the other members of the species complex because it has a different length and sequence of intron.

3.3.4 Analyzing the regulatory region of AsDBLOX gene

To understand the regulation of AsDBLOX gene, we screened its regulatory region or putative transcription factor binding sites with the help of MatInspector and JASPAR software. The search criteria for these transcription factors binding motifs were defined within the insect family. Importantly, three Forkhead, one STAT, two GATA, three C-Myb, one Swi/SNF and one ecdysone transcription factor binding site was predicted by these software (**Figure 3.5**).

Forkhead box- FOX proteins are transcription factors that are reported as crucial mediators of insulin signaling in *Drosophila*, where they regulate cellular growth upon stress conditions like under increased ROS levels (Jünger et al., 2003). They are also important in the *Drosophila* embryonic development (Häcker et al., 1995). The transcription factor STAT is reported to regulate genes involved in mosquito development as well as immunity (Gupta et al., 2009) while GATA is a transcription factor known for its role in hemocyte production in *Chlamys farreri* (Yue et al., 2014) and is also known for tissue-specific expression (Patient and McGhee, 2002). C-Myb is characteristic hemocytes specific hematopoietic transcription factor reported from Pacific oyster *Crassostrea gigas* (Song et al., 2016). In humans, C-Myb functions as a nuclear proto-oncogene (Majello et al., 1986), while in *Drosophila* Myb complex functions in both

activation and repression of DNA replication (Lewis et al., 2004). In *Drosophila*, SWI/SNF chromatin remodeling complex plays an important role in ecdysone-dependent transcription regulation (Tyagi et al., 2009).

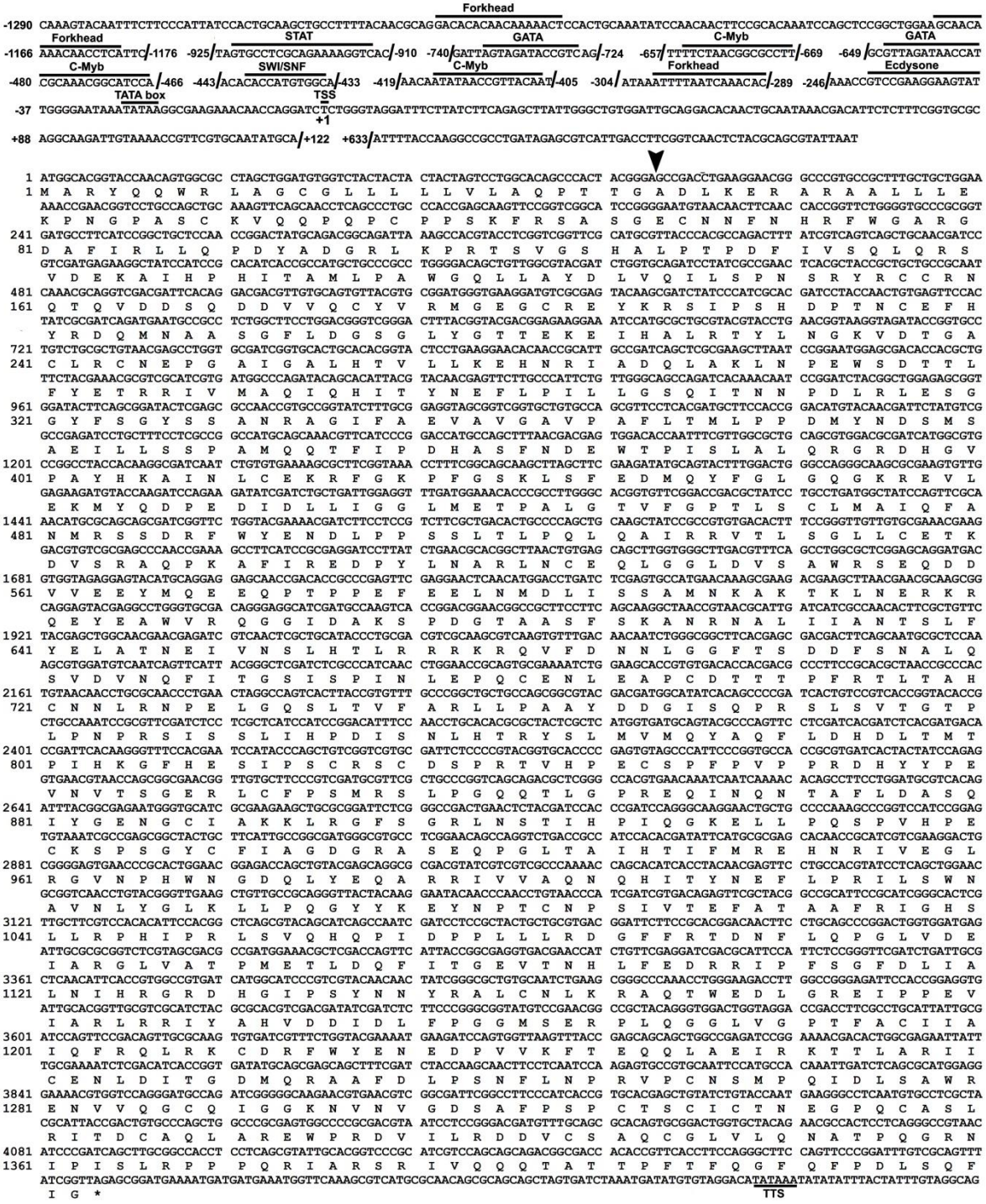


Figure 3.5: Analysis of regulatory region of AsDBLOX. The transcription factors C-Myb, FOXO, GATA, STAT, Ecdysone, SWI/SNF binding sites are indicated in bold. The signal peptide cleavage site is marked by a black arrowhead. The transcriptions start site (TSS), TATA box, transcription termination site (TTS) are also indicated.

3.3.5 Conserved domain analysis of AsDBLOX gene

Based on the conserved regions of AgDBLOX (AGAP008350), we analyzed the predicted AsDBLOX amino acid sequence using Conserved Domain Database (CDD) from NCBI. The putative gene has eight exons with an open reading frame of 4209 bp and codes for a protein of 1402 amino acid residues having molecular weight 161.76

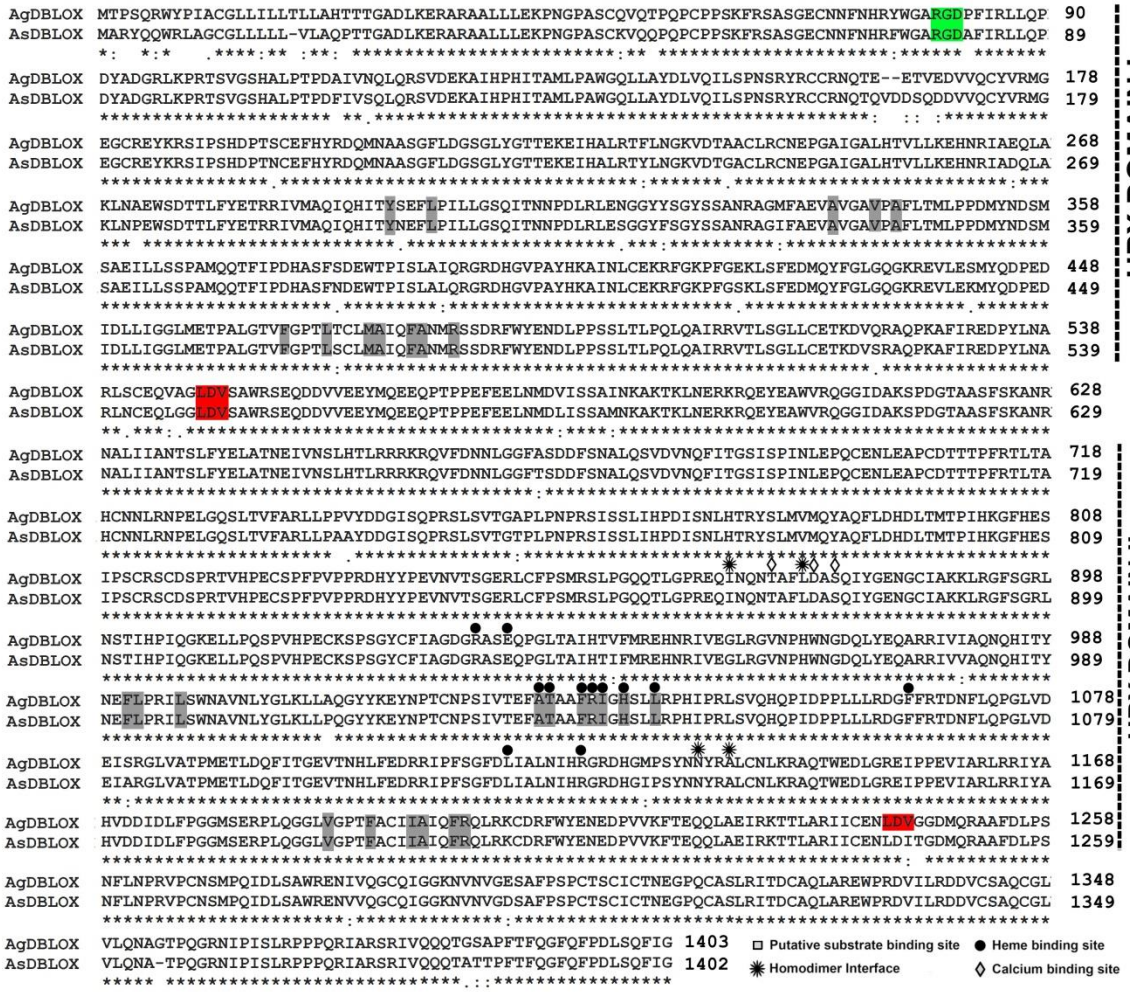


Figure 3.6: Conserve domains in AgDBLOX and AsDBLOX proteins. The conserved domain database (CDD) predicted numerous binding sites such as, heme binding, calcium binding, putative substrate binding and homodimer interface sites as represented by the characteristic symbols in the alignment of predicted full-length AgDBLOX and AsDBLOX proteins. The RGD and LDV integrin binding motifs are highlighted in green and red, respectively.

kDa and theoretical pI of 6.32 (**Figure 3.6**). A noteworthy feature is that both AgDBLOX and AsDBLOX share high amino acid percentage similarity of 96.45%, which means they are true orthologs of each other. Results shown in **Figure 3.6** reveal that the sequence of 4209 nucleotides seems to be divided into two halves and each of them has one putative peroxidase domain (HPX domain I and II). Both HPX domains I and II belong to the animal peroxidase-like superfamily. The HPX domain II has characteristic binding sites such as substrate, heme, and calcium binding sites as mentioned for other

peroxidases based on NCBI CD search. Interestingly, the HPX domain I has only putative substrate binding site with no other binding site as discussed for PDII (**Figure 3.6**). These results indicate that AsDBLOX HPX domain I is not a functional heme peroxidase domain and thus led us to look for its possible role while HPX domain II is a functionally active peroxidase domain. This double peroxidase domain gene is unique in the sense that other peroxidases reported in insects till date have only single peroxidase domain (Kajla et al., 2015).

The Structural Classification of Proteins (SCOP) database defines domains as independent, evolutionary unit that can form a single-domain protein or be part of one or more different multidomain proteins. The domain can either have an independent function or contribute to the function of a multidomain protein in cooperation with other domains (Murzin et al., 1995). The DBLOX is hence a multidomain gene with the two domains possibly serving different functions as the HPX domain I has only substrate binding site and lacks the other features required for a functional heme peroxidase domain. On the other hand, the presence of two integrin binding motifs (**Figure 3.6**) may facilitate extracellular matrix binding of the protein (Johansson and Söderhäll, 1988; Johansson et al., 1995; Lin et al., 2007).

Results depicted in **Figure 3.6** revealed that the DBLOX in both the species exhibits characteristic identity to the peroxinectin-like conserved domain of animal heme peroxidases superfamily because of integrin binding sites. Peroxinectins are secreted as cell-adhesive and opsonic arthropod proteins. They have an integrin binding motif and a peroxidase domain (Johansson and Söderhäll, 1988; Johansson et al., 1995; Lin et al., 2010). Animal heme peroxidases and related proteins superfamily contain a diverse group of enzymes, including peroxidases from metazoans and their members are also found in fungi, plants, and bacteria (Dunford, 1999; Passardi et al., 2007).

3.3.6 AsDBLOX is a secreted globular protein

The secondary structure prediction result of AsDBLOX protein was performed using the Phyre2 online program. It shows that AsDBLOX has 44% α helix (spirals) and 1% β sheets (arrows) (**Figure 3.7**). AsDBLOX was analyzed using SMART program, and the pictorial representation of these results in **Figure 3.8A** revealed that AsDBLOX has two

peroxidase domains labeled as HPX domain I and II, LCR (low complexity region) and also a vWF domain (von Willebrand factor type C domain). The LCR are short stretches of protein sequence with biased amino acid composition. They are found across the protein universe and significantly diverged across protein families. Function for most of them remains to be elucidated (Coletta et al., 2010). The vWF domain is found in various plasma proteins: complement factors B, C2, CR3 and CR4; the integrins (I-domains); collagen types VI, VII, XII, and XIV; and other extracellular proteins. Majority of vWF-

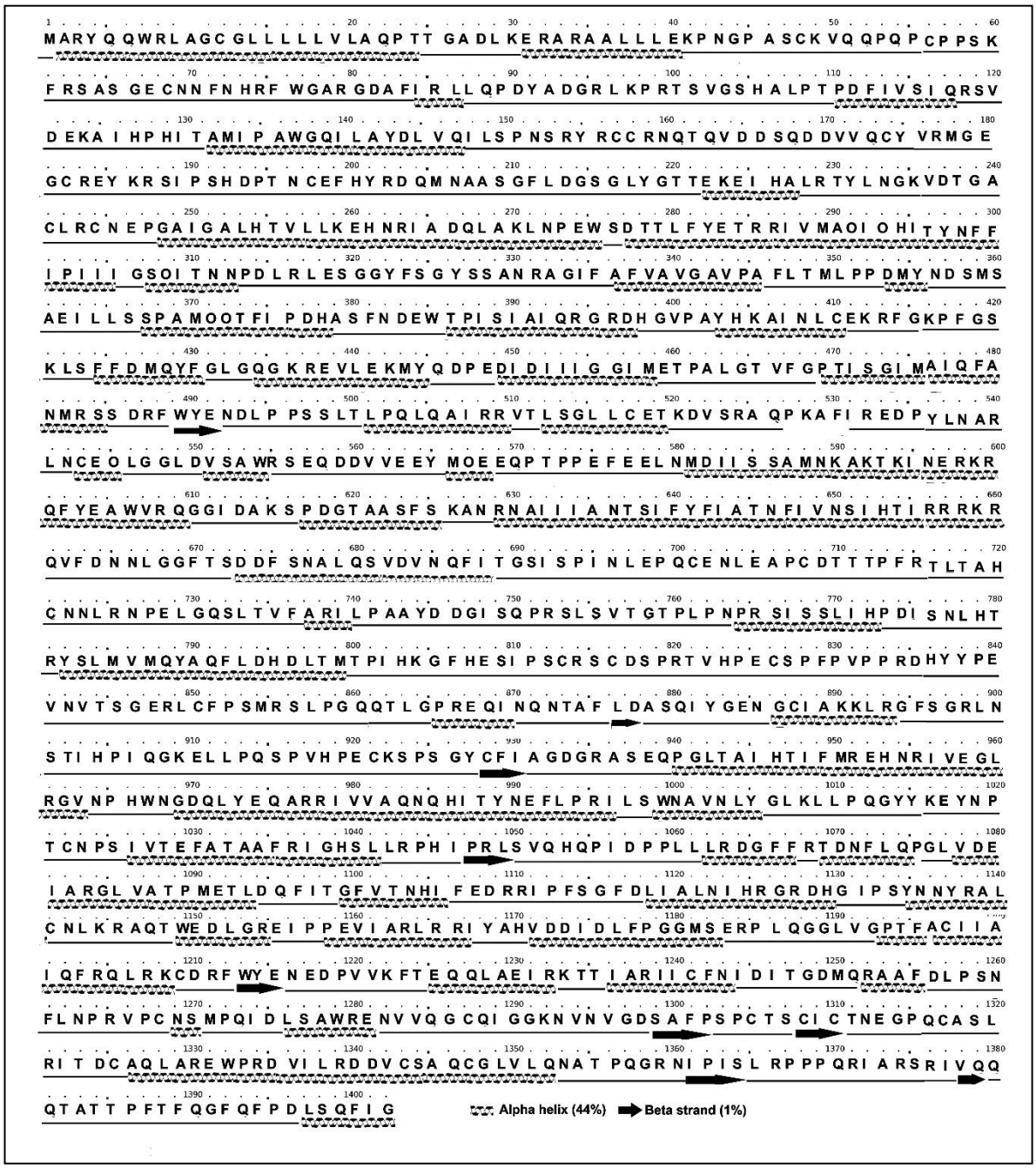


Figure 3.7: Deduced secondary structure of AsDBLOX protein. AsDBLOX protein contains 44% alpha helix (spirals) and 1% beta sheets (arrows) as predicted by Phyre2 software.

containing proteins are extracellular, but some of the most primitive ones present in all eukaryotes are intracellular proteins and are involved in functions such as transcription, DNA repair, ribosomal and membrane transport and the proteasome. A common feature of this domain is that they are reported to be involved in multiprotein complexes. Proteins that incorporate vWF domains participate in numerous biological events (e.g., cell adhesion, migration, homing, pattern formation, and signal transduction), involving interaction with a large array of ligands. The domain is named after the von Willebrand factor (VWF) type C repeat which is found in multidomain protein/multifunctional proteins involved in maintaining homeostasis.

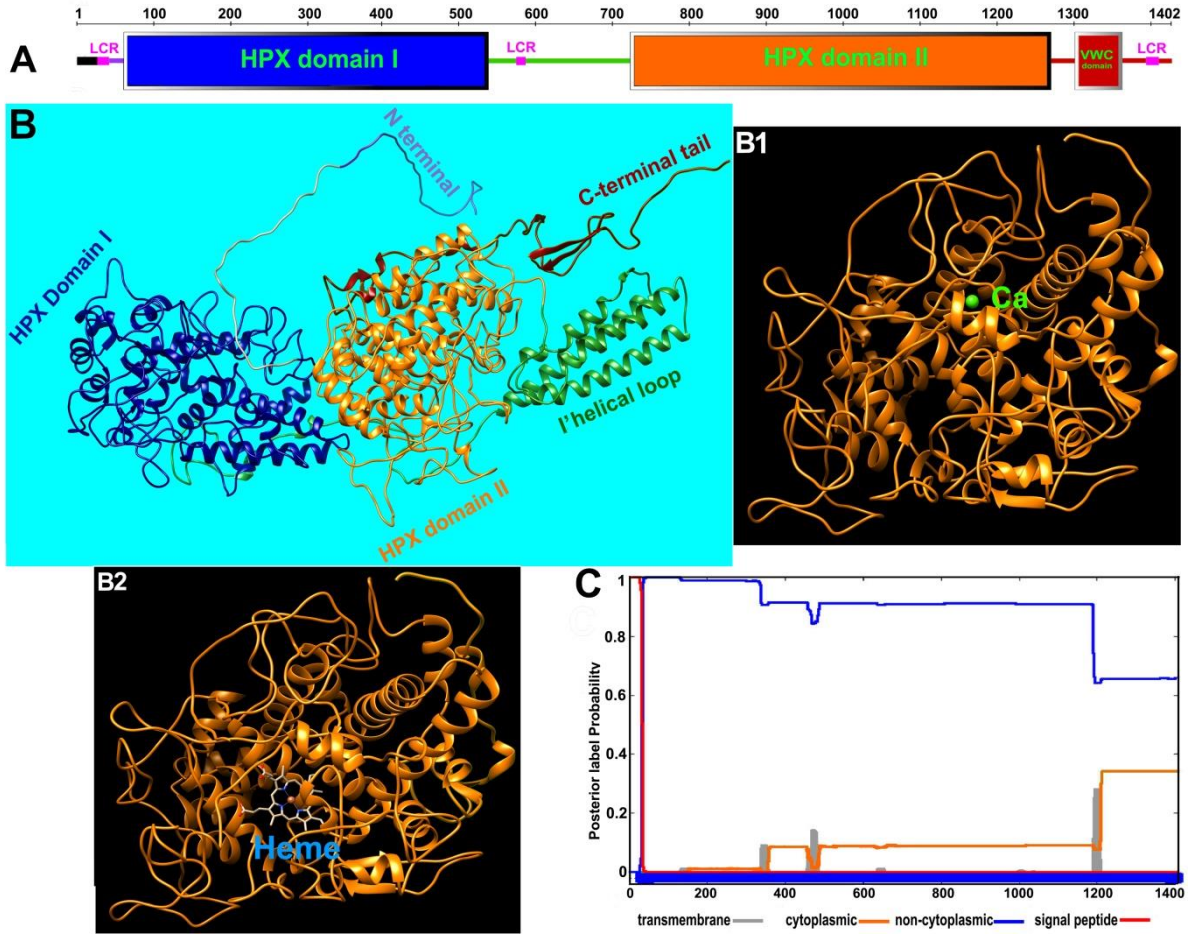


Figure 3.8: ***In silico*** **analysis of AsDBLOX protein**. **A**. SMART prediction of double heme peroxidase domains of ASDBLOX. **B**. 3D structure predicted using Raptorx. The N terminal end is labeled in Violet, HPX domain I in navy blue color, the intermediate beta helical region as green, the HPX domain II as orange and C terminal tail as red. B1 and B2 are Calcium and Heme binding sites predicted in the orange colored HPX domain 2. **C**. The Phobius predicted AsDBLOX to be an extracellular protein.

This is in close similarity to AgDBLOX which has 43% α helix and 3% β sheets. The 3D model of AsDBLOX was generated with the template selected by the RaptorX server, i.e., Myeloperoxidase (5mfaA) with a p-value of 8.57e-23 and an overall uGDT (GDT) of 861 (Källberg et al., 2014; Källberg et al., 2012). The best fit model was selected based on the structure validation servers mentioned in **Materials and Methods** (**Section 2.13**). The Heme ligand binding site (**Figure 3.8 B2**) and Calcium binding site (**Figure 3.8 B1**) are predicted only in the HPX domain 2. The Phobius program predicted the AsDBLOX protein to be completely extracellular (**Figure 3.8C**).

3.3.7 AsDBLOX gene is induced in various stages of mosquito development

Developmental stage-specific expression patterns of AsDBLOX gene was analyzed in all the stages of mosquito development such as eggs, all four stages of larvae, pupae and adult males and females. Semi-quantitative real-time PCR (qPCR) analysis indicated that AsDBLOX is expressed in all these stages of development (**Figure 3.9**). These data indicated that relative mRNA levels of AsDBLOX are least in eggs. In case of 1st instar larvae (LI), the mRNA levels of AsDBLOX increased 4 folds (p= 0.01). Further, the mRNA levels remained elevated 5 fold (p= 0.008) and 7 fold (p= 0.001) during II and III instar stages of larval development respectively, against eggs. However, relative mRNA levels of AsDBLOX gene were lower during LIV stages of larval development (p=0.0019 mRNA levels between eggs and 4th instar

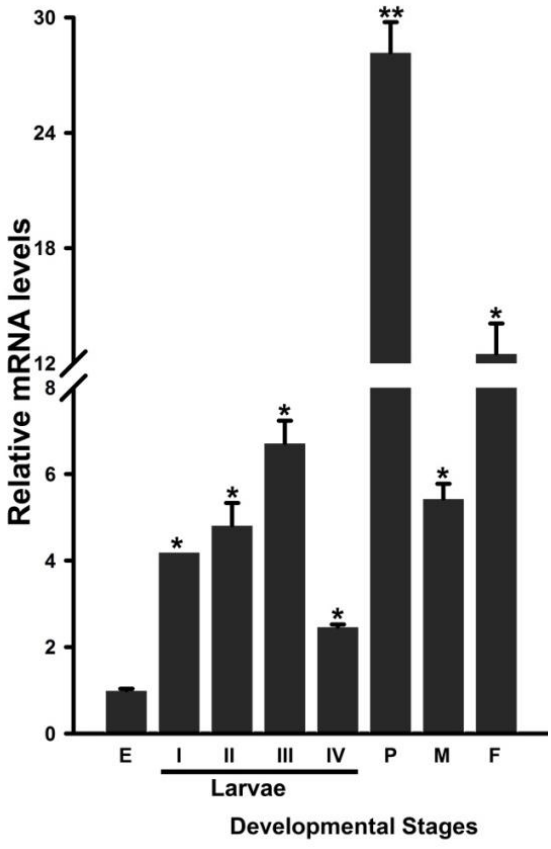


Figure 3.9: Relative mRNA levels of AsDBLOX gene in different developmental stages of *An. stephensi*. Results are representing the mean ± SD of relative mRNA levels of AsDBLOX gene in different developmental stages. The mRNA levels in eggs were considered as control. Significant differences (p < 0.01 or ≤0.05) among relative mRNA levels of different stages against control are indicated by two or one asterisks (*), respectively. E, eggs; I, II, III, IV, various stages of instar larvae; P, pupae; M, males; F, females.

larvae). Interestingly, the expression levels of AsDBLOX increased 30 fold (p=0.003) in pupa in comparison to the eggs (Fig. 3.8).

These results indicated that AsDBLOX gene might have some important role during initial stages of larval development and pupal metamorphosis into adults in a way similar to Cysu, a peroxinectin that is crucial for the wing maturation in Drosophila pupae (Bailey et al., 2017). The comparison of mRNA levels in adult males and females indicated that AsDBLOX expression was 13-fold in females (p= 0.01) and 5-fold in males (p=0.005). This indicated that AsDBLOX may be a female-biased gene. This data is in agreement with previous report of AgDBLOX which showed higher expression in *An. gambiae* females than in males (Marinotti et al., 2006). It is noteworthy to mention here that heme peroxidases are reported both in adult male and female mosquitoes with several roles in physiology, fecundity, survival or immune response (Shaw et al., 2014; Kumar et al., 2010; Kajla et al., 2017a; Kajla et al., 2016). Hence the expression of AsDBLOX gene in the adults can be attributed to one or many of these functions.

3.4 Discussion

The functional units of proteins are domains (they have a distinct structure and function). Our knowledge of the vast proteome is still poor, and the function of many proteins remains to be elucidated. The evolution of a duplicated heme peroxidase is a very intriguing question because its other family members are single domain proteins. Very less literature background is available on DBLOX, which has uniquely two peroxidase domains.

In the present study, we have predicted the genomic organization (**Figure 3.1**) of AsDBLOX. It has eight exons and has a reading frame of 4209 nucleotides which encodes for a protein of 1402 amino acids. We found the presence of an extra intron- I5, in AsDBLOX, which is uniquely absent only in the gambiae complex (**Figure 3.2**). The BLAST result of the AsDBLOX gene revealed its closest match as *An. gambiae* with 100% query coverage, 87% identity and E value of 0.0. Using PCR based approach; we amplified a sequence of 1000bp using F1-R2 primers, and after sequencing (**Figure 3.3**), confirmed DBLOX from *A. stephensi* (**Figure 3.4**). The sequence was confirmed by aligning it with AgDBLOX and predicted AsDBLOX.

To understand the transcriptional regulation of AsDBLOX, we have analyzed its sequence (**Figure 3.5**) and found several crucial Transcription Factor binding sites predicted in its 5 upstream region. The analysis shows that AsDBLOX is probably

regulated by transcription factors like GATA which drive its tissue-specific expression. Also, GATA3 and integrin binding sites are characteristic markers of hemocyte lineage. The ecdysone binding site probably explains its induced expression in pupal stage of development (**Figure 3.8**) as pupa to adult molting is regulated by the ecdysone hormone in insects.

We analyzed the common binding sites shared between the two orthologs AgDBLOX and AsDBLOX (**Figure 3.6**) and found that the HPX domain I has only substrate binding site while the HPX domain II has all the binding sites- Calcium, heme, substrate and homodimer interface. Also, we found one RGD integrin binding site in both AGDBLOX and AsDBLOX, while two LDV integrin binding site in AgDBLOX and one in AsDBLOX. This result points to a dissimilarity between the two orthologs. The secondary structure analysis of AsDBLOX shows that it is a secreted globular protein with 44% helix and 1% beta sheets (**Figure 3.7**). The predicted AsDBLOX was further analyzed by SMART program, and we found that apart from the two heme peroxidase domains, it has regions of low complexity (LCR) and vWF domain (von Willebrand factor type C domain) which are characteristically found in plasma proteins (**Figure 3.8A**). The 3D structure analysis predicted AsDBLOX to have two peroxidase domains with a helical loop spanning between the two domains (**Figure 3.8B**). The Phobius prediction of AsDBLOX showed that it is predicted to be a completely extracellular protein with a clear signal peptide sequence at the beginning of translated protein (**Figure 3.8C**).

AsDBLOX gene is induced in different developmental stages of mosquito such as, larvae and pupae (**Figure 3.9**) which indicates towards its important role in the mosquito development. It is of note that in case of larvae each instar stage is completed with molting that includes the formation of a new cuticle and its sclerotization. This process is governed by ecdysone, a steroidal hormone, which is secreted by the prothoracic glands. In addition, the pupa is the transient stage of mosquito development where metamorphosis takes place, and the role of ecdysone hormone in the regulation of this process is also reported in mosquitoes as well as other insects (Akagi and Ueda, 2011; Telang et al., 2007). Thus, we believe that increased ecdysone activity in larvae and pupae might be one of the factors regulating AsDBLOX gene expression. Our *in silico* analysis of AsDBLOX gene regulatory region revealed the transcription binding sites for ecdysone (**Figure 3.4**) that explain the expression profile of this gene in different developmental stages (**Figure 3.8**). In general, heme peroxidases play an important role in insect development and immunity (Shi et al., 2012). They catalyze tyrosine

crosslinking of proteins, which is a fundamental feature of basal membranes and an elemental mechanism of tissue biogenesis (Péterfi and Geiszt, 2014). In case of the malaria vector, a similar mechanism of peroxidase-mediated crosslinking is also reported in different organs at various stages of development, for example, sclerotization of the cuticle and crosslinking during chorion hardening (Li and Li, 2006).

3.5 Conclusion

In this chapter, we identified and characterized DBLOX from *A. stephensi*. It is a 4209 bp long gene, which has different binding sites in its 5' UTR region and codes for a 1402 amino acids long globular secretory protein. It plays a crucial role in the mosquito development. The role of having multiple integrin binding motifs in this protein, coupled with the fact that its first domain is probably nonfunctional as a peroxidase hint to its possible function as a peroxinectin.

Chapter 4

Tracing the Phylogeny of *An. stephensi* DBLOX: a Bipartite Heme Peroxidase

4.1 Abstract

Here, we attempted to trace the course of evolution and possible function of AsDBLOX – a double heme peroxidase. We found that AsDBLOX is an Ecdysozoa-specific gene. Also, nucleotide BLAST results revealed that the HPX domain I shares no significant similarity with organisms other than mosquitoes, while the protein BLAST result revealed that AsDBLOX protein shares significant similarities with organisms from Phylum Arthropoda and Phylum Nematoda. Further, we found that the HPX domain I (or N terminal HPX domain) had undergone several mutations which resulted in loss of several critical residues (of a functional heme peroxidase) while the HPX domain II (or C terminal HPX domain) was predicted to have all the conserved sites. Hence, we presumed that HPX domain I is non-functional heme peroxidase domain, while HPX domain II is a functional heme peroxidase domain. Moreover, the AsDBLOX HPX domain I was presumed to be under relaxed purifying selection as compared to domain II.

4.2 Introduction

In the previous chapter (**Chapter 3**), we characterized heme peroxidase DBLOX from *An. stephensi*. Heme peroxidases are multigene enzyme superfamily (Zamocky et al., 2015) and the African malaria vector *An. gambiae* has 18 heme peroxidases (HPXs) which are reported to have evolved by gene duplication (Hughes, 2012; Kajla et al., 2015). They all display tissue-specific expression patterns as well have physiological, morphological and immune functions (Kumar et al., 2010; Kajla et al., 2017).

AsDBLOX is a unique gene having two heme peroxidase (HPX) domains – I and II. The HPX domain I has two integrin binding motifs and lack all features of a complete heme peroxidase while HPX domain II is a functional heme peroxidase. Moreover, AgDBLOX gene is reported to have arisen by gene duplication (Waterhouse et al., 2007). Duplication of a gene can have three consequences- a. *non-functionalization* (loss of function due to the accumulation of mutations), b. *neo-functionalization* (NF) (development of a new function) or c. *subfunctionalization (SF)* (both the duplicates undergo mutually complementary degenerative mutations thus acquiring a subfunction of ancestral function) (Ohno, 1999; Force et al., 1999). Hence, gene duplication plays a crucial role in driving evolutionary diversity by supplying functional, redundant DNA sequences as raw materials for evolution.

Our study on heme peroxidase DBLOX – double peroxidase from a major Indian malaria vector, *Anopheles stephensi* revealed a remarkable degree of conservation of amino acid composition despite significant differences in nucleotide sequence between the first and second HPX domain - HPX domain I and II, respectively. For example, the correlation between the percentage amino acid identities was found to be 30.34% while the nucleotide sequences differed from each other at 47.21% of sites. These results imply that by conservation of amino acid at a particular position, there is occurring a substitution codon usage bias in the first domain of this gene. Further studies also show that this gene is under purifying selection in the organisms belonging to Phylum Arthropoda while the gene has undergone positive selection in the Phylum Nematoda. Thus, DBLOX appears to be subjected to an unusual type of purifying selection, where the amino acids are more conserved in the second domain while the first domain has evolved or is still evolving by acquiring mutations. Thus, DBLOX is a classic example of evolution by neo-functionalization.

4.3 Results

4.3.1 Characterization of full-length *Anopheles stephensi* AsDBLOX gene

As described in **Chapter 3**, the full-length CDS of *An. gambiae* DBLOX (AGAP008350) viz. AgDBLOX was not reported in NCBI, and thus we performed an *in silico* full-length gene prediction of both AgDBLOX and AsDBLOX genes. The AsDBLOX gene was predicted to be 5412 bp long with an open reading frame (ORF) of 4206 bp that encodes for a protein of 1402 amino acids.

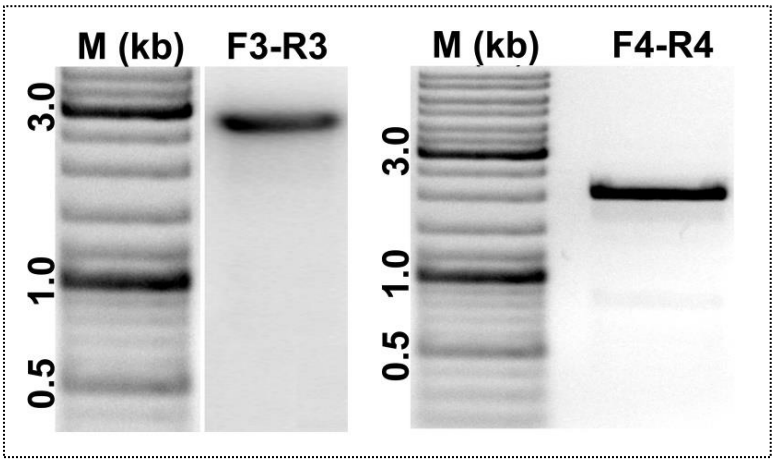


Figure 4.1: PCR amplification of full-length *An. stephensi* AsDBLOX gene. *An. stephensi* cDNA template was amplified using F3-R3 (~3 kb) or F4-R4 (~2.5 kb) primers. The left lane (M), in both the gel images, represents the DNA ladder SM0331 used as reference for identifying the product size.

To amplify full-length AsDBLOX gene, gene-specific primers (mentioned in **Chapter 2**, **Table 2.2**) were designed based on the alignment between AgDBLOX and AsDBLOX sequences and 24 h sugar fed carcass was used as a template (**Figure 4.1B**). The primers

F3-R3 and F4-R4 amplified the expected PCR product of ~3 kb and ~2.5 kb, respectively. The PCR products were sequenced, and BLAST result confirmed that AsDBLOX gene has 98% query coverage and 87% identity (E value 0.0) with its ortholog AgDBLOX gene. The AsDBLOX gene sequence was submitted to the NCBI (GenBank database accession number: MF964191).

4.3.2 AsDBLOX has two duplicated peroxidase domains

4.3.2.1 Nucleotide percentage identity between HPX domain I and II

The full-length AsDBLOX gene was searched for Conserved domains in the NCBI CD database, and we observed that it had two peroxidase domains as discussed in Chapter 3 (**Figure 3.**6). The HPX domain I span from 181-1581 nucleotides while the HPX domain II spans from 2140-3756 nucleotides. The two domains of DBLOX are predicted to be duplicated domains (Hughes, 2012).

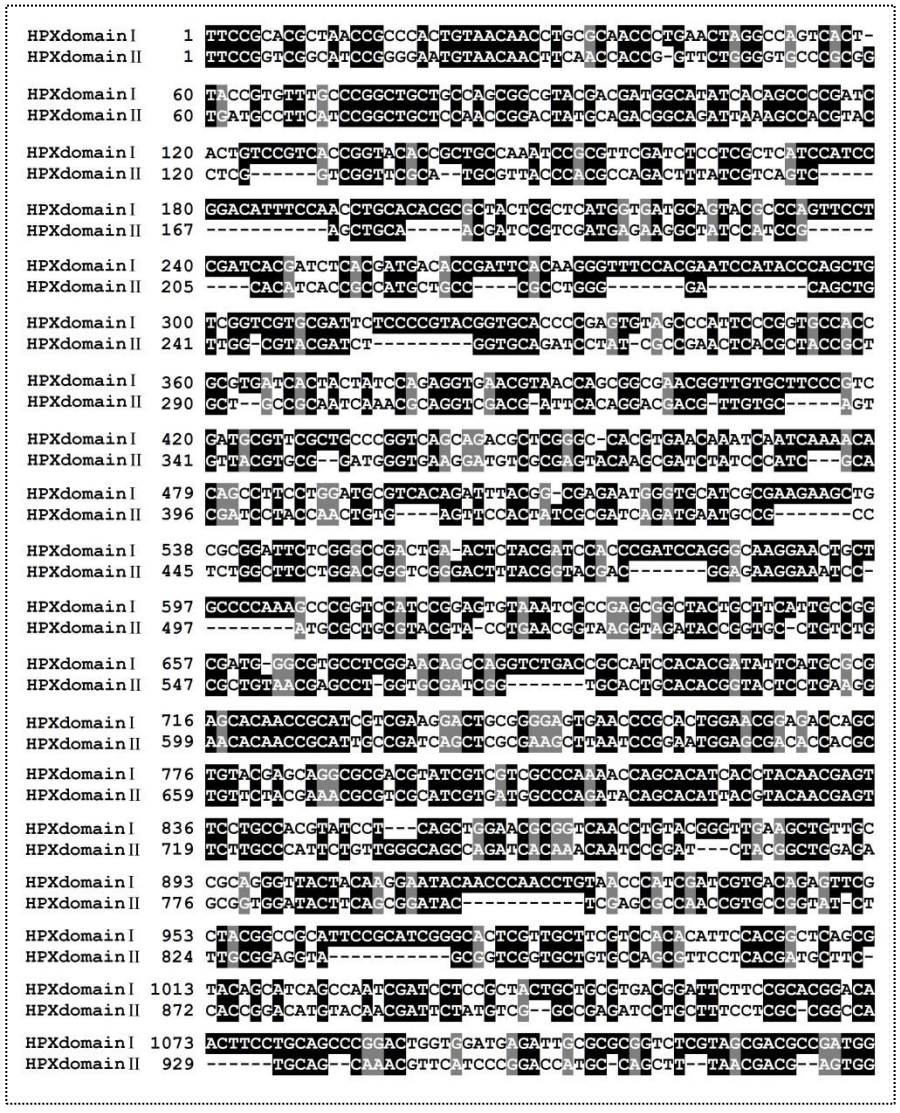


Figure 4.2: Nucleotide alignment of AsDBLOX HPX domain I and II. The degree of similarity between nucleotides ranges from shade - black (maximum similarity), grey (less) to no shading (least). The two domains shared a nucleotide percentage identity of 58.25%.

Furthermore, we looked for the percentage nucleotide similarity between the two domains of AsDBLOX gene. For this, their nucleotide sequences were aligned by Clustal Omega. We found

that the nucleotide percentage identity between them was 58.25%. These findings are in agreement with the previous studies where AgDBLOX was reported to have its two peroxidase domains as duplicates of each other (Hughes, 2012). The multiple sequence alignment presented in **Figure 4.2** was obtained by analyzing the two domains for conserved nucleotides using the Sequence Manipulation Suite at www.bioinformatics.org.

4.3.2.2 Amino acid percentage identity between HPX domain I and II

Furthermore, we tried to find the amino acid percentage similarity between the two domains of AsDBLOX protein. For that, we submitted the sequence of 1402 amino acids of AsDBLOX protein to Conserved Domain Database at NCBI. The HPX domain I was predicted to span between 61 to 527 amino acids and the HPX domain II was predicted to span from amino acid 714 to 1252 amino acids. These two domains were aligned using Clustal Omega program, and we observed that they share an amino acid identity of 30.34%. The AgDBLOX was previously reported to have internally duplicated two heme

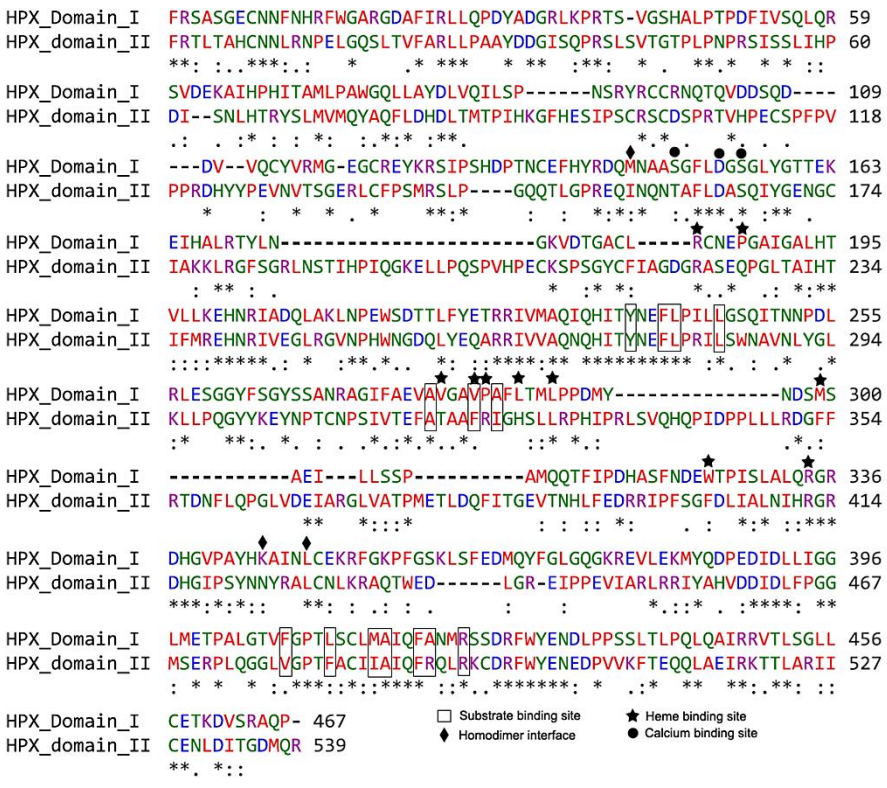


Figure 4.3: Amino acid alignment of HPX domain 1 and II. The two domains share an amino acid percentage identity of 30.34% as determined by Clustal Omega. The figure shows the marked conservedness in amino acids between the HPX domain I and II for the substrate binding sites (rectangular boxes). While the amino acids for heme binding(star), calcium binding (circle) and homodimer interface (prism) are present only in the HPX domain II and these amino acids are absent in the respective positions at HPX domain I.

peroxidase domains (Waterhouse et al., 2007; Hughes, 2012).

4.3.3 The HPX domain I is an incomplete peroxidase domain

Our next approach was to compare the conserved amino acids between the AsDBLOX HPX domain I and II (**Figure 4.3**). We observed that the two domains shared common amino acids for substrate binding sites (**Figure 4.3**). On the other hand, the motifs for heme binding, calcium binding, and homodimer interface were absent in the HPX domain I (**Figure 4.3**). This implied that there have been major mutational changes in the HPX domain I, that led to its possible loss as a "complete" heme peroxidase domain. Such examples are also observed in other proteins where duplication of one domain occurred during evolution. Interestingly, one of the domains became non-functional. However, another domain remains functional. For example catalase-peroxidase (katG) protein from bacteria, fungi, and archaebacteria. In this protein, there occurred duplication of peroxidase domain. The N-terminal peroxidase domain underwent mutations and functions as a catalase. The C terminal peroxidase domain however, underwent deleterious mutations that might made it non-functional (Smulevich et al., 2006; Zámocký and Obinger, 2010). The loss of the functionality in one domain is achieved by mutations as we observed in the case of AsDBLOX (Zámocký and Obinger, 2010).

Calcium	**(TCT)**		**(GAC)**		**(TCG)**					
	S_{149}		**D_{153}**		**S_{155}**					
	T_{160}		**D_{164}**		**S_{166}**					
	(ACA)		**(GAT)**		**(TCA)**					
Homodimer Interface			**(ATG)**		**(AAG)**		**(CTG)**			
			M_{145}		**K_{345}**		**L_{349}**			
			I_{156}		**N_{423}**		**L_{427}**			
			(ATC)		**(AAC)**		**(CTG)**			
Heme	**(CGC)**	**(CCT)**	**(GTC)**	**(GTG)**	**(CCA)**	**(CTC)**	**(CTT)**	**(ATG)**	**(TGG)**	**(CGT)**
	R_{163}	**P_{187}**	**V_{280}**	**V_{283}**	**P_{284}**	**L_{287}**	**L_{290}**	**M_{299}**	**W_{325}**	**R_{334}**
	R_{222}	**Q_{226}**	**T_{319}**	**F_{322}**	**R_{323}**	**H_{326}**	**L_{329}**	**F_{353}**	**F_{403}**	**R_{412}**
	(CGT)	**(CAG)**	**(ACG)**	**(TTC)**	**(CGC)**	**(CAC)**	**(CTT)**	**(TTC)**	**(TTC)**	**(CGT)**

Figure 4.4: Comparison between amino acid residues corresponding to mutations in codons in the HPX domain I (black) and II (red). The two domains were compared for finding the mutations in HPX domain I which led to its loss of residues required for forming a complete functional heme peroxidase domain.

4.3.4 The HPX domain I of AsDBLOX gene is conserved in mosquitoes

Out of the 18 heme peroxidases reported in *An. gambiae*, only one is a domain duplicated heme peroxidase, i.e., DBLOX. Hence, we performed the phylogenetic analysis of this

gene, to elucidate its evolution. For this, the full-length AsDBLOX gene was BLAST searched against general nucleotide sequences database. Surprisingly, we observed that the full-length DBLOX gene was present only in the mosquitoes- Ag (*An. gambiae*), Aae (*Aedes aegypti*), Cq (*Culex quinquefasciatus*) (**Figure 4.5**). Moreover, the query coverage dropped drastically for other insects, for instance *Drosophila melanogaster* (Dm) (**Figure 4.5**)(less than 60%) (**Table 4.1**), which showed that the double HPX domain gene architecture was present only in mosquitoes.

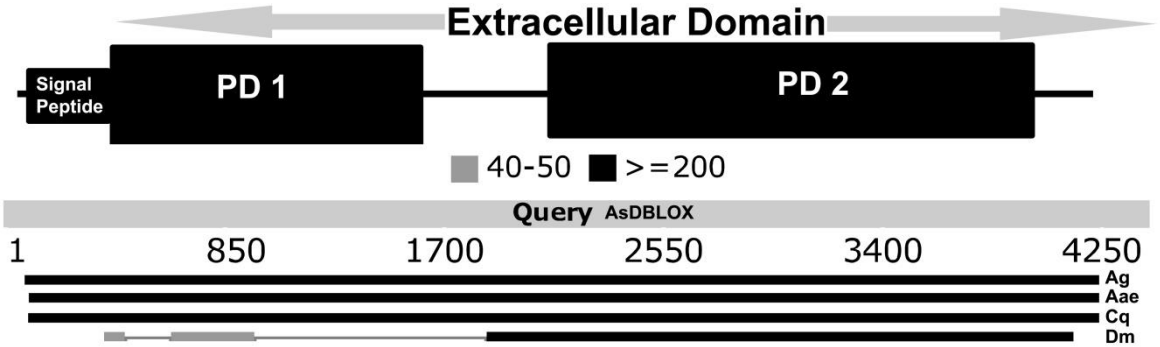


Figure 4.5: Nucleic acid similarity of AsDBLOX with Ag (*An. gambiae* DBLOX), Aae (*Aedes aegypti* DBLOX), Cq (*Culex quienquifasciatus* DBLOX), Dm (*Drosophila melanogaster* DBLOX) of HPX domain 1 and domain II. As depicted in **Figure 4.5**, the nucleotide similarity of DBLOX is almost insignificant with *Drosophila* whereas the mosquitoes have high percentage similarity.

Table 4.1: Nucleotide BLAST results of AsDBLOX. The BLAST results revealed that apart from *Anopheles gambiae*, *Culex quinquefasciatus and Aedes albopictus*, which have 95% query coverage, other insects have query coverage of less than 62%, i.e. only HPX domain II. This indicated that the two domains of DBLOX are present only in mosquitoes.

List of peroxidases from various organisms	Max score	Total score	% Query	E value	% Identity	Accession
Anopheles gambiae AGAP008350-PA DBLOX	4982	5182	95%	0.0	87	XM_317106.5
Culex quinquefasciatus oxidase/peroxidase	3171	3337	95%	0.0	77	XM_001862001.1
Aedes albopictus uncharacterized	2877	3040	95%	0.0	76	XM_019684601.1
Drosophila melanogaster CG10211	1050	1384	54%	0.0	73	NM_001299114.1
Bactrocera dorsalis	1047	1399	61%	0.0	72%	XM_011211792.2
Dinoponera quadriceps	1036	1224	47%	0.0	72%	XM_014614608.1
Rhagoletis zephyria myeloperoxidase	1034	1138	56%	0.0	71%	XM_017630121.1
Cerapachys biroi	1005	1123	56%	0.0	71%	XM_011337493.2
Musca domestica	1003	1109	54%	0.0	70%	XM_005179676.3
Bactrocera cucurbitae uncharacterized	987	1248	62%	0.0	72%	XM_011195952.1
Nasonia vitripennis peroxinectin	937	1040	47%	0.0	71%	XM_016983810.1
Ceratitis capitata peroxidasin	926	1040	53%	0.0	71%	XM_004531265.2
Wasmannia auropunctata	892	952	44%	0.0	71%	XM_011702600.1
Trichogramma pretiosum	888	1099	55%	0.0	71%	XM_014382381.1

Vollenhovia emeryi	872	983	45%	0.0	71%	XM_012018087.1
Linepithema humile peroxidase-like protein	870	1029	51%	0.0	71%	XM_012361912.1
Orussus abietinus uncharacterized	854	1011	54%	0.0	69%	XM_012428858.1
Megachile rotundata uncharacterized	841	841	44%	0.0	70%	XM_003705528.2
Pseudomyrmex gracilis uncharacterized	830	882	44%	0.0	70%	XM_020435471.1
Stomoxys calcitrans uncharacterized	830	986	50%	0.0	70%	XM_013244973.1
Papilio polytes peroxidasin	746	805	45%	0.0	70%	XM_013288237.1
Cephus cinctus uncharacterized	737	838	52%	0.0	69%	XM_015742465.1
Solenopsis invicta uncharacterized	737	783	46%	0.0	69%	XM_011157918.1
Spodoptera exigua POX-H	693	750	35%	0.0	72%	KJ995809.1
Neodiprion lecontei uncharacterized	681	681	44%	0.0	68%	XM_015655876.1

4.3.5 Amino acid conservation of two domains of AsDBLOX

The full-length AsDBLOX protein of 1402 amino acids was subjected to PSI-BLAST (Position-Specific Iterative Basic Local Alignment Search Tool) in the NCBI database. It derives a position-specific scoring matrix (PSSM) or profile from the multiple sequence alignment of sequences detected above a given score threshold using protein-protein BLAST. Thus, PSI-BLAST provides a means of detecting distant relationships between proteins.

The BLAST results (**Figure 4.6**) revealed that AsDBLOX shared significantly high amino

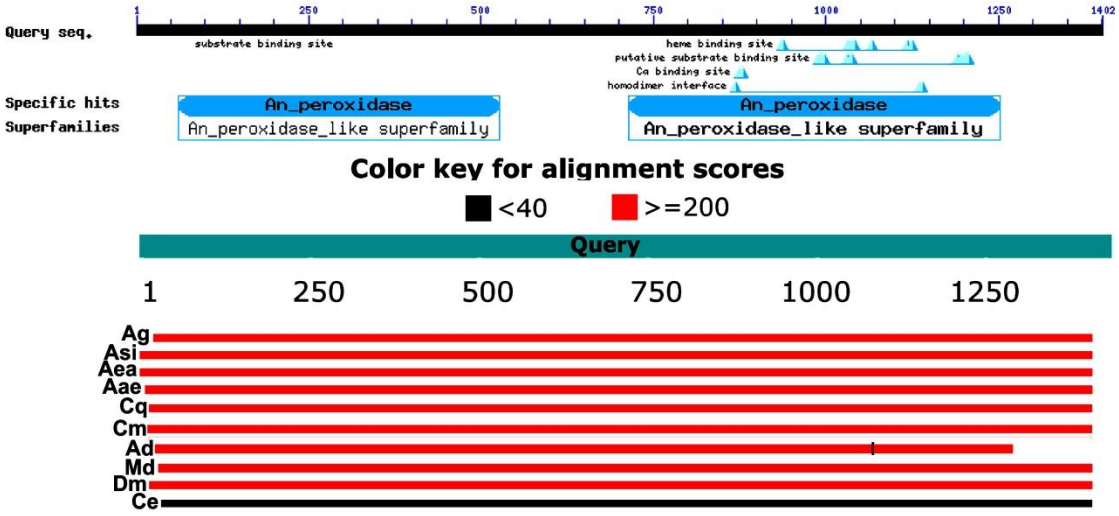


Figure 4.6: AsDBLOX protein is present in different organisms. The top of the figure shows the AsDBLOX Conserved domains result obtained from PSI-BLAST. The below picture shows pictorial representation of selected organisms obtained from PSI-BLAST - Ag (*An. gambiae* DBLOX), Asi (*An. sinensis* DBLOX), Aea (*Aedes albopictus* DBLOX), Aae (*Aedes aegypti* DBLOX), Cq (*Culex quienquifasciatus* DBLOX), Cm (*Clunio marinus* DBLOX), Ad (*An. darlingi* DBLOX), Md (*Musca domestica* DBLOX), Dm (*Drosophila melanogaster* DBLOX), Ce (*Caenorhabditis elegans*). The amino acid similarity of DBLOX full length polypeptide is predicted to be present in almost all the

acid query coverage for the selected organisms – 95% in Ag (*An. gambiae*), 97% in Asi (*An. sinensis*), 97% in Aea (*Aedes albopictus*), 96% in Aae (*Aedes aegypti*), 96% in Cq (*Culex quienquifasciatus*), 96% in Cm (*Clunio marinus*), 87% in Ad (*An. darlingi*), 95% in Md (*Musca domestica*), 93% in Dm (*Drosophila melanogaster*), 90% in Ce (*Caenorhabditis elegans*). However, the nucleotide query coverage was comparatively very less in the genes of other insects, for example- 55% in Dm (*Drosophila melanogaster*), 51% in *Spodoptera exigua* (Se), and 56% in *Musca domestica* (Md). These results revealed that there is amino acid conservedness of DBLOX across these groups of organisms.

4.3.6 DBLOX is an Ecdysozoa specific gene

To analyze the presence of DBLOX gene in other organisms, we performed a phylogenetic analysis of the protein. Briefly, the results obtained from PSI-BLAST were retrieved in FASTA format. We obtained Superphylum Ecdysozoa with members from Phylum Arthropoda and Nematoda from PSI-BLAST results. The phylogenetic tree was prepared in MEGA7.0 using PSI-BLAST results and manually selected sequences (**Materials and Methods**, **Table 2.3)**. The multiple sequence alignment (MSA) of the 51 selected sequences were subjected to the maximum-likelihood (ML) method of the MEGA7.0 package with the Jones Taylor Thornton (JTT) model of amino acid substitution, pairwise deletion of gaps. The confidence of phylogeny was conducted with the bootstrap method; by setting the bootstrap replications to 1000 as before (Kajla et al., 2015). Heme peroxidases from *Arabidopsis thaliana* (outgroup) and *Homo sapiens* (Hs) were also selected for the analysis. All the organisms of Phylum Arthropoda, Nematoda formed their respective single nodes. The phylogenetic analysis indicates that DBLOX is possibly an Ecdysozoa specific gene. The superphylum Ecdysozoa forms a clade of the molting animals (Aguinaldo et al., 1997; Dunn et al., 2008). Ecdysozoa is a clade comprising of eight phyla: Arthropoda, Tardigrada, Onychophora (have segmentation and appendages), Nematoda, Nematomorpha, Priapulida, Kinorhyncha and Loricifera (worms with an anterior proboscis or introvert) (Chapman et al., 2009). Moreover, peroxinectins reportedly belong to the peroxidase-cyclooxygenase family of animal heme peroxidases, and they are specific for Ecdysozoans (Zamocky et al., 2008).

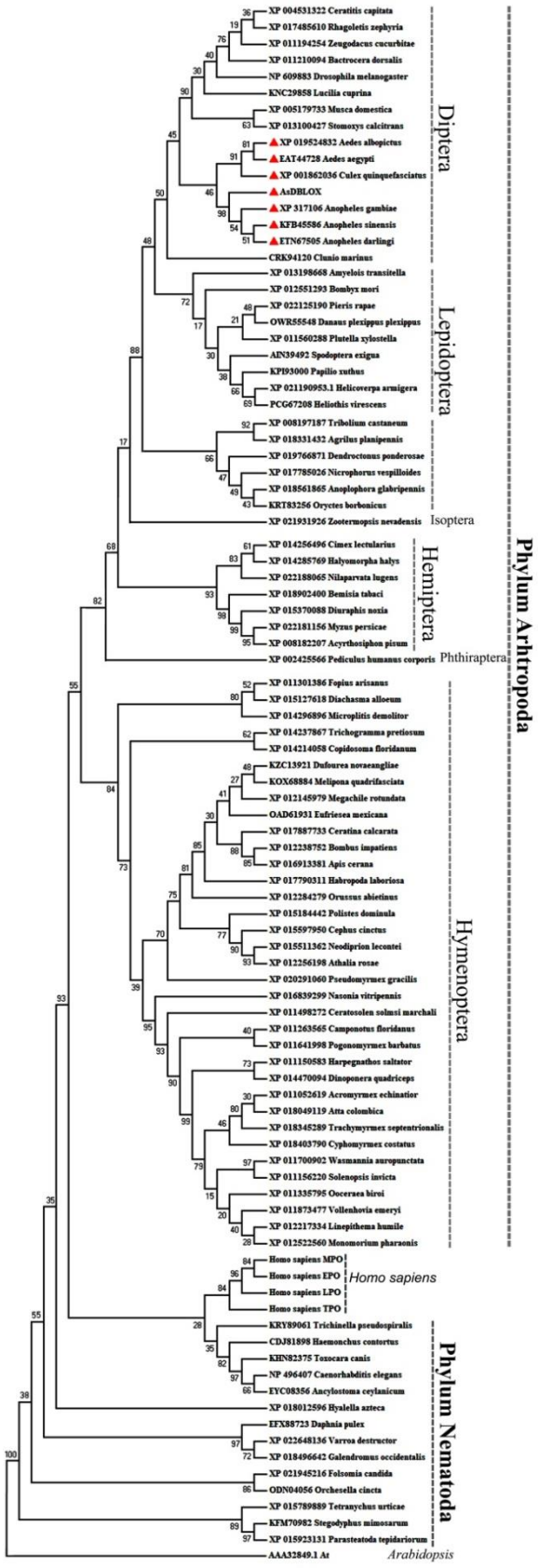


Figure 4.7: Extensive phylogenetic analysis of AsDBLOX across the Kingdom Animalia. Sequences retrieved from PSI-BLAST result were retrieved from NCBI. The red triangles represent the mosquitoes. Also, sequences of Human heme peroxidases (MPO, EPO, LPO, TPO) and plant *Arabidopsis* were manually selected. The phylogenetic analysis was performed using MEGA7.0 maximum likelihood with 1000 bootstrap replicates. The list of organisms is mentioned in **Table 2.4**.

4.3.7 Analysis of selection pressure on AsDBLOX

As discussed before, AsDBLOX gene is a result of gene duplication. Hence, we wanted to explore the selection pressure on the two domains. Briefly, the selection pressure on protein-coding genes is assessed by two quantities dN (rate of nonsynonymous substitutions per nonsynonymous site, also called Ka) and dS (rate of synonymous substitutions per synonymous site, also called Ks). A Ka/Ks ratio more than 1 implies positive or balancing selection, while a ratio less than 1 implies negative or purifying selection. While the former is plastic and free to evolve, the latter is constrained and highly optimized by natural selection and thus has a low tolerance for changes. (Nielsen and Yang, 1998; Suzuki and Gojobori,

1999; Hurst, 2002; Koonin and Rogozin, 2003). But synonymous substitutions are not neutral selection. Selection aims to increase the translational efficiency and accuracy which shapes codon usage bias in organisms ranging from bacteria to mammals (Ikemura, 1981; Sharp and Li, 1986; Akashi, 1994; Stoletzki and Eyre-Walker, 2006; Drummond and Wilke, 2008; Higgs and Ran, 2008). Other selection pressures at synonymous sites are exerted towards splicing (Chamary and Hurst, 2005a; Warnecke and Hurst, 2007), DNA and messenger RNA (mRNA) secondary structure, stability (Vinogradov, 2003; Chamary and Hurst, 2005b; Stoletzki, 2008).The following

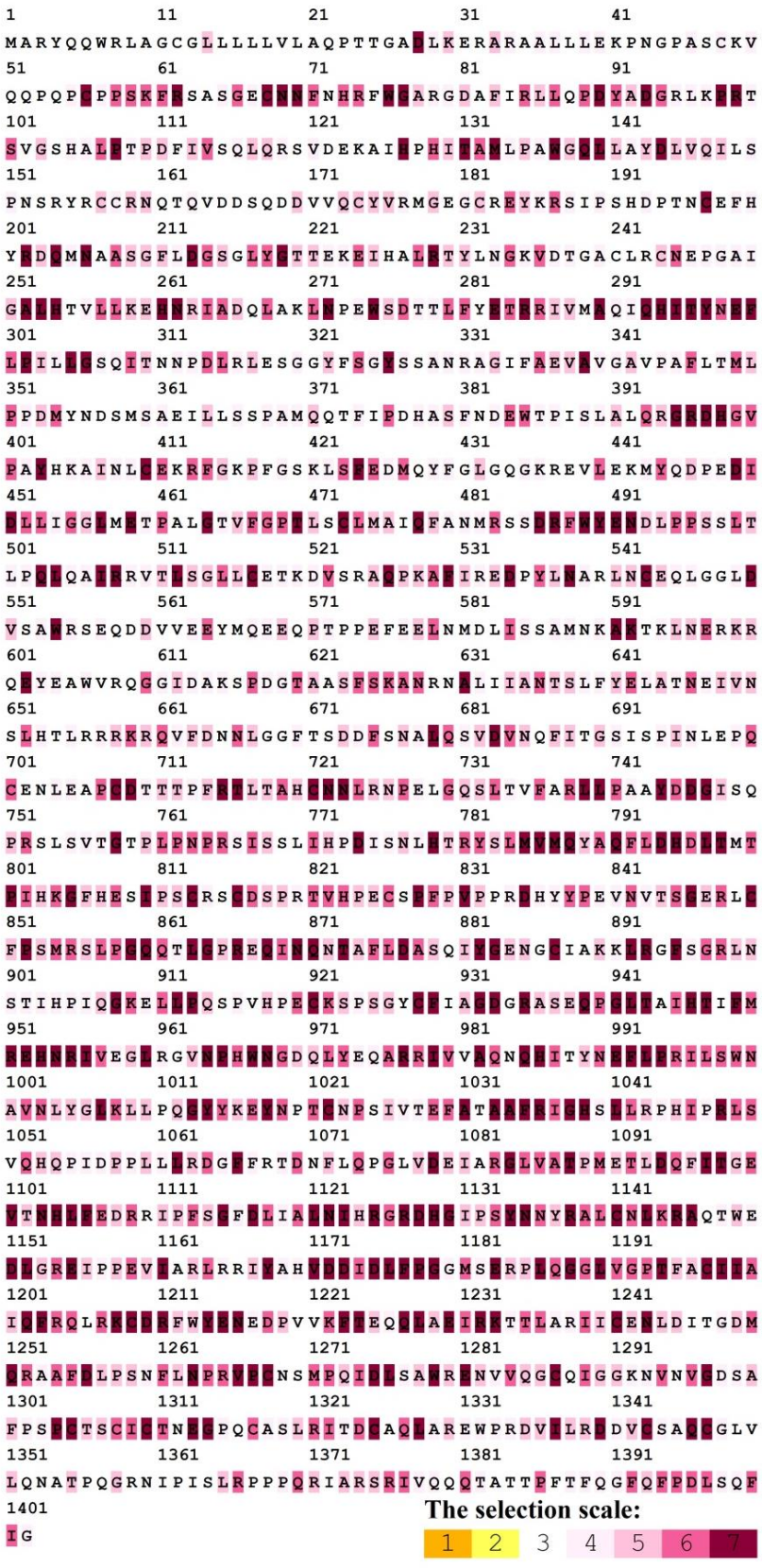


Figure 4.8: Purifying selection pressure on AsDBLOX protein. The majority of the amino acids in HPX domain II are under stringent purifying selection, while the amino acids in HPX domain I have relaxed selection pressure.

putative DBLOX sequences - Dm10211 (*Drosophila melanogaster*), Dpu EFX88273 (*Daphnia pulex*), Cel (*Caenorhabditis elegans*) and the reported mosquito DBLOX sequences-AsDBLOX, AgDBLOX, Aae AAEL003933 (*Aedes aegypti*), Cq XM_001862001 (*Culex quienquifasciatus*)- were submitted to http://services.cbu.uib.no/tools/kaks server and it showed that majority of HPX domain II amino acids are under stringent purifying selection (Figure 4.8). On the contrary, the amino acid residues comprising the HPX domain I have comparatively less purifying selection pressure. In fact, there were a significant percentage of amino acids in HPX domain I, which lied under the neutral selection. This implied that the HPX domain I has undergone mutations in DNA which is reflected in its low purifying selection score. There also lies a possibility that during the further course of evolution, the first domain may acquire new functions, i.e., *neo-functionalization*. There is also a report which states that genes encoding extracellular proteins in mammals and fishes are preferentially targeted by positive selection (Montoya-Burgos, 2011). This study also reported that a high proportion of genes which contain both positively selected motifs and neutral evolving regions have neutrally-evolving regions as the major source of novelties that are screened by natural selection and hence may lead to positive selection (Montoya-Burgos, 2011).

Zhang et al. (2007) reported that insects have specific proteins like stress response, stimulus-response proteins, specific cuticle and pheromone/odorant binding proteins. These proteins distinguish the evolution of insects from other eukaryotes. Thus, they tend to have low *Ka/Ks* ratio which suggests purifying selection pressure. Also, a large number of paralogs in insect-specific proteins possibly indicate towards their adaptation to environmental changes (Zhang et al., 2007).

4.3.8 Analysis of *Ka/Ks* ratio in DBLOX gene from various organisms

The evolution of multigene heme peroxidase superfamily in organisms is complex as they reportedly formed by gene duplication and have evolved to perform multiple functions. Hence, they exist under a strong purifying selection pressure, so that random mutations are not incorporated. Purifying selection is a very restrictive form of evolution. There exists several examples in insects where we found that many insect-specific genes are under purifying selection pressure such as vitellogenin genes in mosquito (Chen et al., 2010), smell and taste receptors in *Drosophila* (McBride, 2007), PGRP(PeptidoGlycan Receptor Protein), an immune gene for parasite recognition in *Drosophila* (Jiggins and Hurst, 2003).

Moreover, DBLOX is a domain duplicated gene, and it is widely reported that duplication results in one partner that experiences lesser functional constraints and hence accumulates mutations at an accelerated rate (Lynch and Conery, 2000). Hence, we were interested in analyzing selection pressure on DBLOX gene and thus we selected DBLOX amino acid sequences from representative organisms of each taxonomic group - Dm10211 (*Drosophila melanogaster*), Dpu EFX88273 (*Daphnia pulex*), NP_496407 Cel (*Caenorhabditis elegans*) and the reported mosquito DBLOX sequences-AsDBLOX, AgDBLOX, Aae AAEL003933

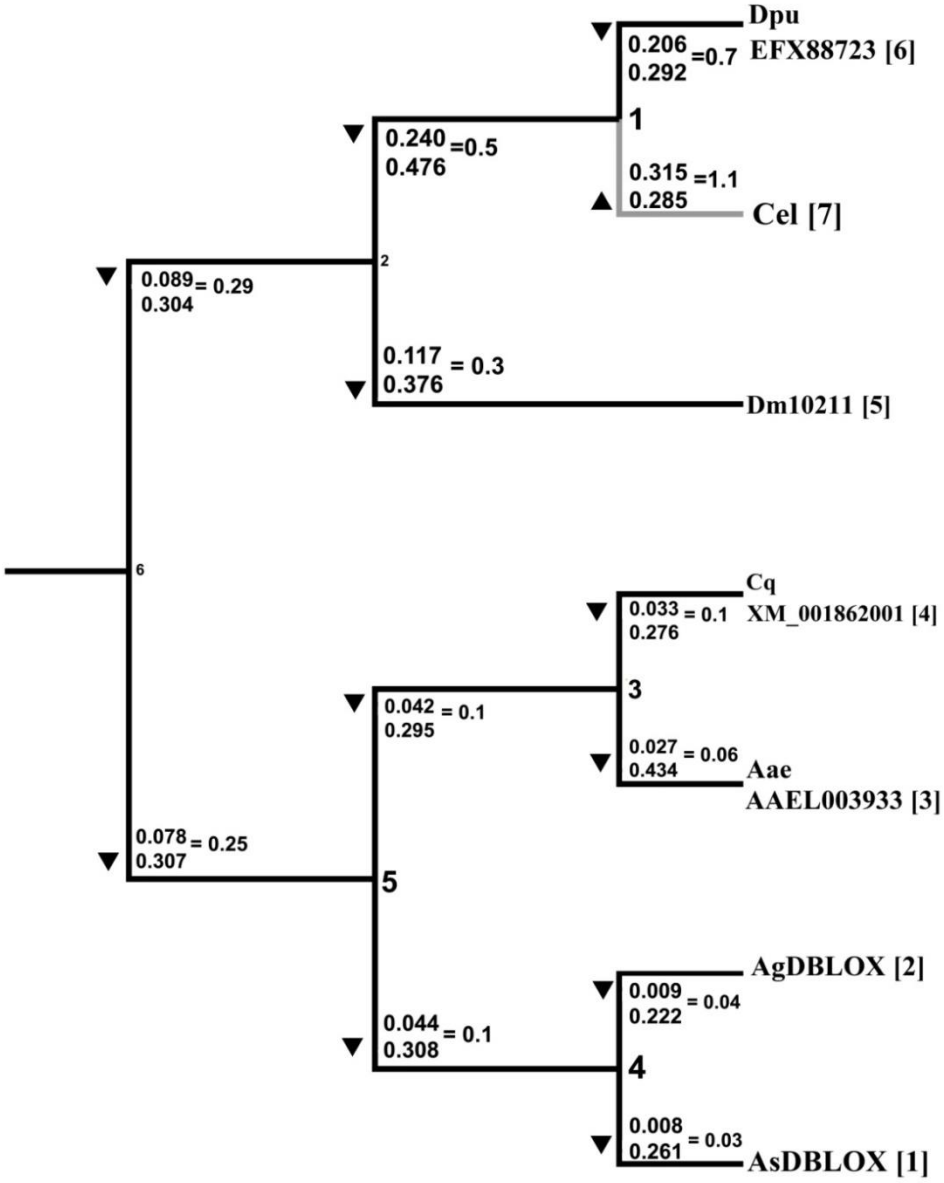


Figure 4.9: *Ka/Ks* ratios of selected organisms representing each major group. AsDBLOX [1], AgDBLOX AGAP008350 [2], Aae AAEL003933 (*Aedes aegypti*) [3], Cq XM_001862001 (*Culex quienquifasciatus*) [4]- all mosquitoes. Dm10211 (*Drosophila melanogaster*) [5], Dpu EFX88273 (*Daphnia pulex*) EFX88723 [6], Cel (*Caenorhabditis elegans*) NP_496407 [7]. The downward arrowhead shows Ka/Ks ratio <1 i.e. purifying selection, while the upward arrowhead shows viceversa.

(*Aedes aegypti*), Cq XM_001862001 (*Culex quienquifasciatus*). The results presented in **Figure 4.9** shows that putative AsDBLOX homolog in *C. elegans* has undergone positive selection (Ka/Ks ratio >1) as compared to the other Arthropods (*An. gambiae*, *A. aegypti*, *C. quinquefasciatus*, *D. melanogaster and D. pulex*) (Ka/Ks ratio <1) where the DBLOX protein has possibly undergone negative or purifying selection. Hence, DBLOX protein is highly diverged among the Arthropods and Nematodes.

4.3.9 AsDBLOX orthologs are present in all major malaria vectors

The putative DBLOX peroxidase contigs (or Ensembl identifiers) were retrieved from the genome of different anophelines using nucleotide sequence of full-length predicted

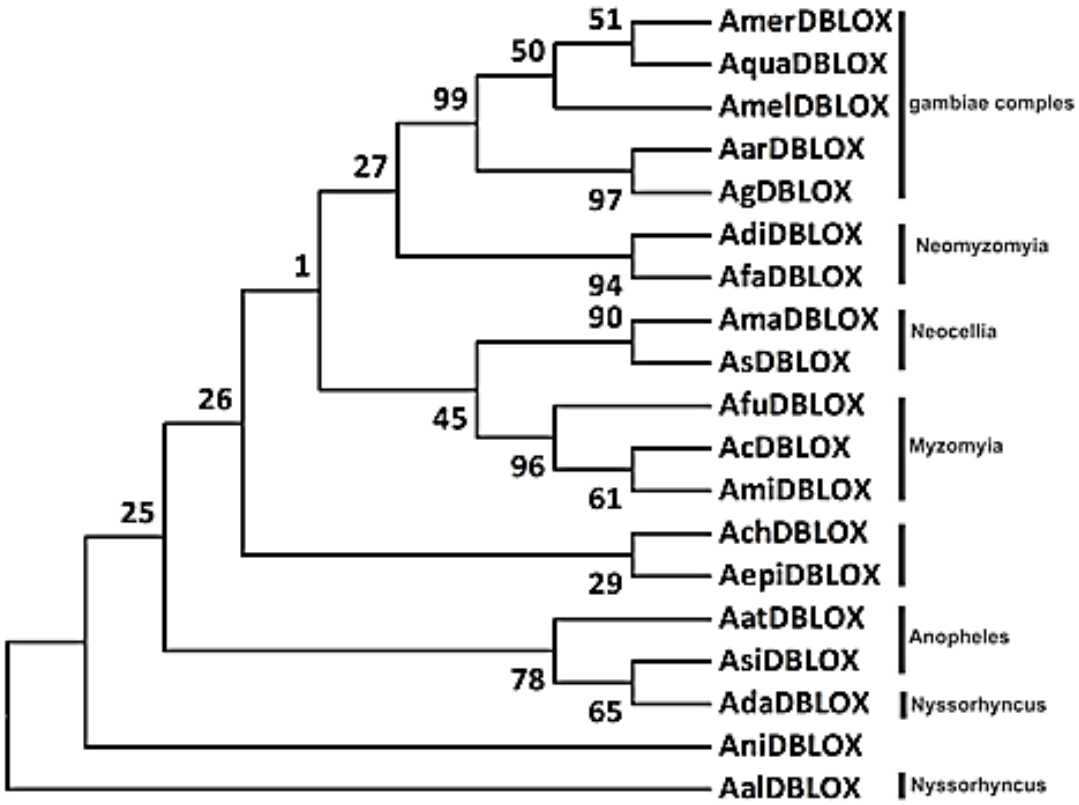


Figure 4.10. Phylogenetic analysis of DBLOX across worldwide distributed anophelines. The tree was generated using Maximum Likelihood with 1000 bootstraps in MEGA 7.0.

AsDBLOX as a query. DBLOX contig and total amino acids in DBLOX protein are shown in the table (**Table 3.3**). The abbreviations for individual *Anopheles* species are mentioned in parenthesis.

We found that all of these *Anopheles* species have the putative DBLOX gene in their genome. Interestingly, in this comprehensive analysis of 19 anophelines (see **Table 3.2**) there were 12 major, 5 minor and 2 nonmalaria vector species as defined by others (Neafsey et al., 2013). Hence, the presence of DBLOX-like peroxidases in the genome of vectors and non-vector species indicated towards DBLOX having some possible role in the development and physiology (**Chapter 3**). This can be hypothesized based on the fact that DBLOX belongs to the peroxinectin group of heme peroxidases. Peroxinectins belong to the peroxidase-cyclooxygenase superfamily and are characterized by an integrin binding motif and a peroxidase domain. In freshwater crayfish *Pacifastacus leniusculus*, peroxinectin is synthesized and stored in hemocytes in an inactive form, which is released during degranulation response activated upon pathogen recognition, and activated outside the cells to mediate hemocyte attachment and spreading for encapsulation of the pathogen (Johansson and Söderhäll, 1988; Liang et al., 1992).

Table 4.3: List of putative DBLOX peroxidases retrieved from different species of *Anopheles* mosquitoes. The putative DBLOX peroxidase contig (or GenBank accession no.) from the genome of different anophelines were retrieved using nucleotide sequence of AsDBLOX (GenBank: MF964191) as query. The overhanging sequences of the contigs were trimmed based on AsDBLOX sequences as mentioned in the Materials and Methods. The total amino acids in trimmed sequences are presented in the table. The abbreviation for individual mosquito is also mentioned in parenthesis.

Vectorial capacity	*Anopheles* spp. (abbreviation)	Gene ID/ GenBank acc. No./Contig	DBLOX protein (amino acids)
Major vectors	*An. arabiensis* (Aar)	1.786	1393
	An. atroparvus (Aat)	1.391	1373
	An. darlingi (Ada)	1798	1387
	An. dirus (Adi)	1.1599	1403
	An. farauti (Afa)	2.807	1397
	An. funestus (Afu)	1.2057	1400
	An. maculatus (Ama)	1.14744	819
	An. sinensis (Asi)	2.649	1389
	An. nili (Ani)	25684	562
	An. gambiae (Ag)	AGAP008350	1382
	An. culicifacies (Ac)	1.2012	1403
	An. stephensi (As)	1690	1444
Minor vectors	*An. albimanus* (Aal)	1.289	867
	An. epiroticus (Aep)	1.3594	1402
	An. melas (Amel)	2.15946 2.26187	1038
	An. merus (Amer)	2.1295	1406
	An. minimus (Ami)	1.1993	1395
Non-vectors	*An. quadriannulatus* (Aq)	1.1072	1406
	An. christyi (Ach)	1.2049	1389

Further, we compared the predicted protein sequences of all the putative DBLOX sequences to analyze their relative identity with AgDBLOX and AsDBLOX. The amino acids identity among these DBLOX peroxidases was found to be highly conserved (84-99%, **Table 4.3**).

4.3.10 The DBLOX gene is continuously evolving

The multiple sequence alignment between all the retrieved *Anopheles* sequences obtained from protein BLAST results (**Table 2.4**) revealed some previously unreported features. Peroxinectins are extracellular heme peroxidases, and they have an integrin binding motif in addition to the heme peroxidase domain. RGD and LDV are well-known motifs for integrin binding report widely (Zhang et al., 2014). All the DBLOX sequences have single RGD and LDV motif in the predicted HPX domain I. We also observed another LDV/I motif in the HPX domain II of all *Anopheles* species including AsDBLOX (**Figure 4.11**).

The evolution of basal lineages of *Anopheles* appears to have diversified rapidly in association with the separation of South America, Africa, and Laurasia ~120–95 Mya (Krzywinski et al., 2001; Nishihara et al., 2009). The Afrotropical *Anopheles gambiae* complex comprises of closely related and morphologically indistinguishable mosquito species which differ dramatically in their capacity to transmit malaria, ranging from major vectors (*An. gambiae*, *An. arabiensis*) through minor or locally important vectors (*An. merus*, *An. melas*) and nonvectors (*An. quadriannulatus, An. christyi*) (White et al., 2011). Diversification of the *An. gambiae* complex and ongoing diversification within its nominal species appears to be a product of recent and rapid adaptation to environmental heterogeneities, notably those of anthropogenic origin. The *An. gambiae* complex has an LDI motif while the other *Anopheles* mosquitoes have LDV motif. These differences in orthologs of DBLOX prompted us to hypothesize that this gene is continuously evolving and is probably acquiring new features in due course of evolution (Neafsey et al., 2015).

Table 4.2 Amino acid Percentage identity matrix of DBLOX in all major malaria vectors. The percentage identity for the DBLOX protein between different Anophelines is presented in a tabular format with the values in green highlighted boxes similar to that of blue boxes.

Sl.no	*Anopheles*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	AalDBLOX		95.07	84.65	85.42	83.31	84.96	86.21	85.35	85.63	85.65	85.02	85.73	84.62	84.75	84.52	84.1	84.73	93.15
2	AdDBLOX	95.07		87.49	88.39	85.28	88.1	88.28	87.67	88.84	87.97	88.08	88.74	88.1	88.01	88.22	87.18	87.61	95.68
3	AniDBLOX	84.65	87.49		94.92	90.27	91.7	92.26	92	91.98	91.9	92.11	92.26	91.68	91.7	91.41	90.98	91.09	95.33
4	AatDBLOX	85.42	88.39	94.92		90.05	91.2	92.41	92.06	91.69	91.96	91.68	91.97	91.82	92.04	91.47	91.25	91.37	95.67
5	AmaDBLOX	83.31	85.28	90.27	90.05		95.13	96.37	97.2	94.82	95.84	95.23	94.4	95.12	95.12	94.92	93.94	94.24	98.37
6	AfuDBLOX	84.96	88.1	91.7	91.2	95.13		96.85	95.71	95.42	96.63	95.56	85.2	94.84	95.06	94.77	94.25	94.45	97.83
7	AmiDBLOX	86.21	88.28	92.26	92.41	96.37	96.85		97.21	96.14	98.28	96.34	96.13	96.28	96.2	95.99	95.41	95.61	98.17
8	AsDBLOX	85.35	87.67	92	92.06	97.2	95.71	97.21		95.71	96.28	96.2	95.85	96.35	95.78	95.71	95.13	95.41	98.17
9	AdiDBLOX	85.63	88.84	91.98	91.69	94.82	95.42	96.14	95.71		95.62	95.63	98.21	95.56	95.63	95.7	95.12	95.32	98.33
10	AcDBLOX	85.65	87.97	91.9	91.96	95.84	96.63	98.28	96.28	95.62		96.05	95.4	95.48	95.43	95.7	94.63	94.42	98.33
11	AchDBLOX	85.02	88.08	92.11	91.68	95.23	95.56	96.34	96.2	95.63	96.05		95.56	96.92	97.07	96.92	96.43	96.57	98.5
12	AfaDBLOX	85.73	88.74	82.26	91.97	94.4	95.2	96.13	95.85	95.21	95.4	95.56		95.34	95.7	95.63	95.12	95.39	98.67
13	AepDBLOX	84.62	88.1	91.68	91.82	95.12	94.84	96.28	96.35	95.56	95.48	96.92	95.34		97.07	97	96.51	96.72	98.67
14	AmerDBLOX	84.75	88.01	91.7	92.04	95.12	95.06	96.2	95.78	95.63	95.43	97.07	95.7	97.07		99.07	99.06	98.99	100
15	AgDBLOX	84.52	88.22	91.41	91.47	94.92	94.77	95.99	95.71	95.7	95.7	96.92	95.63	97	99.07		98.84	98.98	100
16	AquaDBLOX	84.1	87.18	90.98	91.25	93.94	94.25	95.41	95.13	95.12	94.63	96.43	95.12	96.51	99.06	98.84		98.77	100
17	AarDBLOX	84.73	87.61	91.09	91.37	94.24	94.45	95.61	95.41	95.32	94.42	96.57	95.39	96.72	98.99	98.98	98.77		100
18	AmelDBLOX	93.15	95.68	95.33	95.67	98.37	97.83	98.17	98.17	98.33	98.33	98.5	98.67	98.67	100	100	100	100	

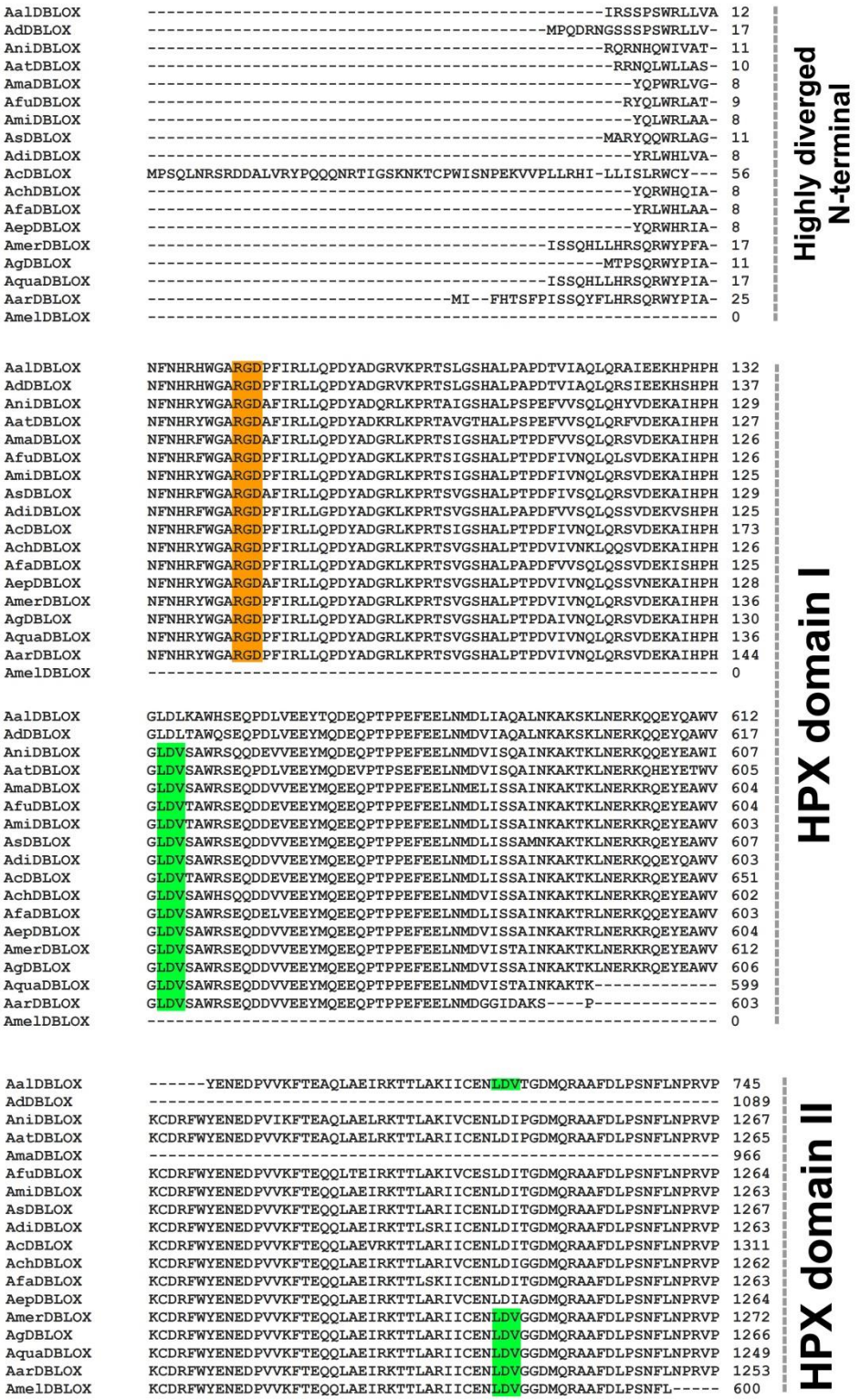


Figure 4.11: Amino acid sequence alignment of DBLOX from different *Anopheles* species. It shows conserved integrin binding motifs – RGD (red) and LDV (green). Moreover, *An. merus*, *An. gambiae*, *An. quadriannulatus*, *An. arabiensis*, *An. melas* and *An. albimanus* have a LDI motif in HPX domain II while other *Anopheles* species have LDI motif.

4.4 Discussion

The immune system has two branches- acquired and innate. While the acquired branch evolved ~500mya in vertebrates (Meyer and Zardoya, 2003; Takezaki et al., 2003; Pancer and Cooper, 2006), the innate branch is present in both implying that it is the ancestral form of immunity. Because of their importance in recognition of pathogens and their elimination which ultimately aid in the insect survival, there exists Insects possess only innate immunity, but their prevalence on this planet since time millions of years ago makes them ideal candidates to study their molecules whether they belong to physiological, morphological, developmental or immune pathways. Heme peroxidases (HPXs) are a multigene family of enzymes that mainly catalyze the one- and two-electron oxidation of many organic and inorganic substrates where the redox cofactor - heme b or post-translationally modified heme, gets ligated to either histidine or cysteine residues. Four heme peroxidase superfamilies (peroxidase-catalase, peroxidase-cyclooxygenase, peroxidase-chlorite dismutase and peroxidase-peroxygenase) have evolved independently (Zamocky et al., 2015). Peroxinectins belong to the peroxidase-cyclooxygenase superfamily. They have an integrin binding motif and a peroxidase domain.

AsDBLOX has two integrin binding motifs- RGD and LDV, present within its two peroxidase domains. Hence, it is possibly a peroxinectin- like molecule that mediates adhesion of hemocytes to extracellular matrix. The HPX domain I is not conserved in nucleotide while it is conserved in amino acids indicating synonymous codon usage. It also indicated that the first domain had undergone nucleotide base mutations when compared to the HPX domain II which has more conserved nucleotides and amino acids. We observed that DBLOX is a unique Ecdysozoa specific gene, i.e., it is present in members of Phylum Arthropoda and Nematoda. These results (Figure are in agreement with a previous report by Zámocký and Obinger (2010) that peroxinectins are found in members of Ecdysozoa. The similarity with any other phylum of Kingdom Animalia was not significant. We found no orthologous genes or proteins of DBLOX in other Phylum of Kingdom Animalia through our General BLAST or PSI-BLAST. This indicated the presence of DBLOX only in members of Superphylum Ecdysozoa. Also, the HPX domain I has comparatively lesser pressure of purifying selection as compared to HPX domain II. It has probably undergone several mutations in the course of evolution from its MRCA (most recent common ancestor). The DBLOX phylogeny of major *Anopheles* vectors revealed that the African *An. gambiae*

complex has another LDV motif (in HPX domain II) which is different from other worldwide *Anopheles* sp. that have LDI motif. Moreover, the N terminal of this gene is highly diverged among the anophelines.

4.5 Conclusion

An. stephensi AsDBLOX is a duplicated heme peroxidase. The two domains have possibly evolved by duplication and the HPX domain I has relaxed selection pressure. Moreover, the African *An. gambiae* complex has further diverged from the other worldwide distributed anophelines. Hence, DBLOX is a unique gene and is an example of two domains with different selection pressures.

Chapter 5

Identification of a unique role of AsDBLOX in mosquito hemocyte immunity

5.1 Abstract

Here, we carried out the functional characterization of heme peroxidase DBLOX from Indian malaria vector *An. stephensi*. AsDBLOX gene expression was relatively high in sugar-fed female mosquitoes and was downregulated in midguts or carcasses post 24 h of blood feeding, or *P. berghei* infected blood feeding. We found that AsDBLOX is a peroxinectin, i.e., hemocytes-specific gene and is synthesized by the hemocytes. Surprisingly, the gene was induced in *Plasmodium*-infected blood fed carcasses at 3h, in comparison to the control blood-fed carcasses. Thus, *Plasmodium*-infected blood meal induces mosquito hemocyte immunity in the carcass. The AsDBLOX gene was also induced in bacteria-challenged *An. stephensi* larvae and adults. The silencing of AsDBLOX gene in *An. stephensi* adults increased the bacterial population in comparison to the control dsLacZ injected mosquitoes. Hence, AsDBLOX possibly plays a crucial role in mosquito anti-bacterial and anti-plasmodial immune responses and thus, can be exploited in anti-malarial strategies.

5.2 Introduction

Cellular processes like aerobic respiration generate free radicals for instance O_2^- as byproducts which have damaging effects on the biomolecules and ultimately body tissues (Betteridge, 2000). Superoxide dismutases (SOD), catalases and heme peroxidases (HPXs) play a fundamental and an indispensable role in maintaining the antioxidant protective capacity of insects (Felton and Summers, 1995) and, any imbalance between the Reactive Oxygen Species (ROS) and antioxidant defenses leads to the generation of oxidative stress (Betteridge, 2000). Mosquitoes, throughout their life cycle, thrive in microbe-rich environments. Moreover, their hematophagous (blood feeding) nature exposes them to additional oxidative stress (high levels of heme released during blood digestion).

Hemocyte generated ROS-mediated oxidative burst (mediated by ROS) is used by hemocytes to control invading pathogens in insects. It also regulates the differentiation of hemocytes (Owusu-Ansah and Banerjee, 2009). Peroxinectin is a type of heme peroxidase that is synthesized and stored in hemocytes in an inactive form and is activated by microbial compounds like lipopolysaccharide, β-1, 3-glucan, and peptidoglycan (Lin et al., 2007). They are characterized by a peroxidase domain and an

integrin-binding motif - RGD (Arg – Gly – Asp) / KGD (Lys –Gly – Asp) / LDV (Leu – Asp – Val). Thus, peroxinectin function in hemocyte cell adhesion and migration, as well as display heme peroxidase activity (Johansson et al., 1995; Sritunyalucksana et al., 2001; Dong et al., 2009; Liu et al., 2004b; Yang et al., 2015). Also, they are implicated in the activation of the prophenoloxidase (PPO) cascade for eliminating microorganisms in cooperation with extracellular superoxide dismutase (EC-SOD) (Holmblad and Söderhäll, 1999; Johansson et al., 1999).

AsDBLOX is a peroxinectin having two peroxidase domains as well as integrin binding motifs (as discussed in Chapters 3 and 4). In this chapter, we studied the role of HPX - AsDBLOX in mosquito hemocyte immunity. It is unique double peroxidase gene having two domains possibly serving two different functions thus illustrating an example of gene duplication with the evolution of multidomain proteins. Our results revealed that DBLOX is a hemocytes specific gene and it is significantly induced by bacterial challenge in both *An. stephensi* larvae as well as adults. Moreover, this gene is also induced in the *P. berghei* infected mosquito carcasses which indicate the activation of mosquito hemocyte immunity against the parasite. Silencing of AsDBLOX gene induced the growth of bacteria indicating an anti-bacterial role of this gene.

5.3 Results

5.3.1 Mosquito body compartments specific expression of AsDBLOX gene

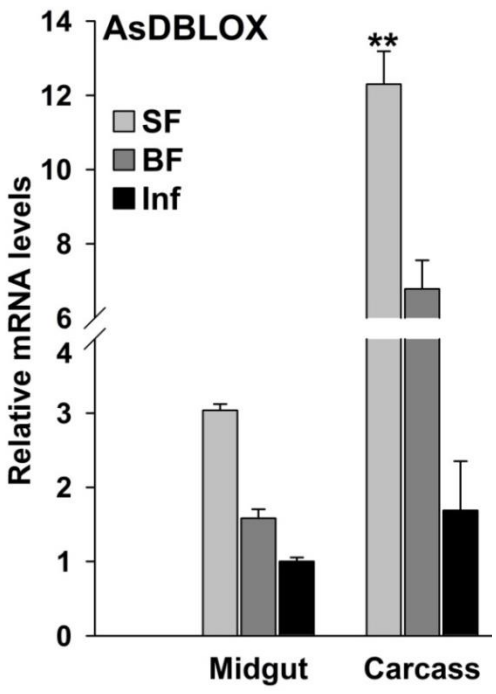


Figure 5.1: Expression of AsDBLOX gene in different body compartments of *An. stephensi*. Relative mRNA levels of AsDBLOX gene were analyzed in midguts and carcasses of sugar fed (SF), 24h post uninfected- (BF) or *P. berghei* (Inf) infected-blood fed female mosquitoes. Significant difference $p \leq 0.001$ in relative mRNA levels of AsDBLOX is indicated by two asterisks (*).

Initially, we analyzed the relative mRNA levels of AsDBLOX gene in sugar-fed (SF), 24 h post uninfected- (BF) or *P. berghei* infected- blood fed (Inf) mosquito midguts or carcasses (rest of the body). The 24 h post fed samples were selected because 24 h corresponds to the time point when ookinetes invade the

midgut epithelium and induce mosquito immunity (Smith et al., 2014). Results presented in **Figure 5.1** revealed that the basal levels of AsDBLOX mRNA were ~4-fold higher in sugar-fed carcasses than sugar fed midguts (p=0.0092). The gene expression was downregulated in blood fed as well as *Plasmodium*-infected mosquito midguts by ~2 fold and ~3-fold, respectively, against sugar fed midguts. Also, the gene expression was downregulated by ~2-fold ($p \leq 0.05$) and 7-fold (p=0.0107) in blood fed and *Plasmodium*-infected carcasses when compared to sugar fed carcasses.

These results indicated that AsDBLOX gene exhibits maximum basal expression levels in sugar-fed midguts or carcasses. The down-regulation of this gene in both these body compartments after control of infected blood feeding (**Figure 5.1**) was in contrast to the previous findings of Kumar et al. (2004) where they reported the induction of *An. gambiae* AgDBLOX gene in *Plasmodium*-infected midguts.

5.3.2 AsDBLOX is a hemocytes-specific gene

Based on our previous results discussed in Chapters 3 and 4, AsDBLOX is a peroxinectin. These kinds of proteins are synthesized and secreted by the granulocytes (a type of hemocytes) (Smith et al., 2016). Thus, we hypothesized that AsDBLOX is a hemocyte-specific gene and further analyzed the hemocytes-specific expression of this gene. We isolated hemocytes from mosquitoes following the method as discussed in **Materials and Methods** and compared the expression of AsDBLOX gene in midguts, carcasses, and hemocytes as shown in **Figure 5.2**.

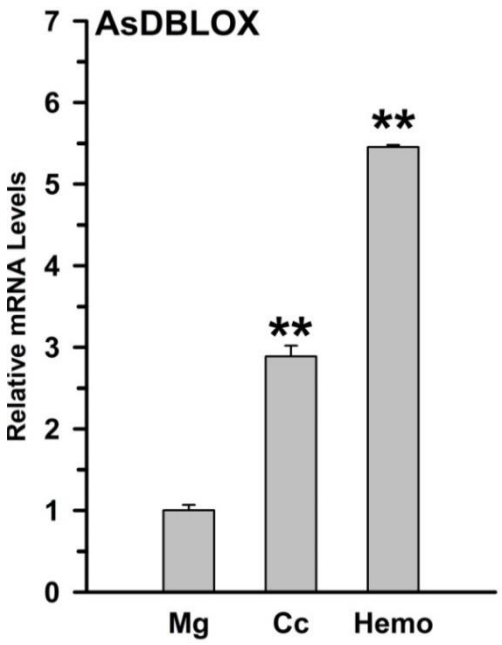


Figure 5.2: Tissue specific expression of AsDBLOX gene. Relative mRNA levels of AsDBLOX were analyzed in sugar-fed midguts (Mg), carcasses (Cc) and hemocytes (Hemo). Significant differences $p \leq 0.001$ in relative mRNA levels of AsDBLOX gene is indicated by two asterisks (*).

The expression of AsDBLOX was ~3-fold (p=0.0059) and ~6-fold (p=0.002) higher in sugar-fed carcasses and hemocytes, respectively when compared to the sugar-fed midguts. This indicated that AsDBLOX is largely induced in hemocytes.

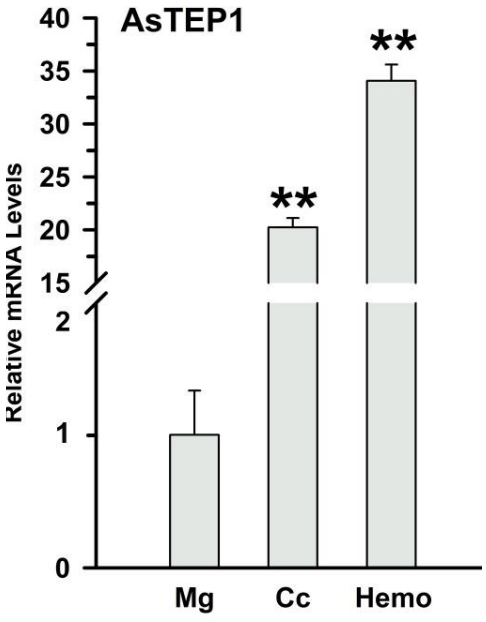


Figure 5.3: Tissue specific expression of AsTEP1 gene. Relative mRNA levels of AsTEP1 were analyzed in sugar-fed midguts (Mg), carcasses (Cc) and hemocytes (Hemo)**.** Significant differences $p \leq 0.001$ in relative mRNA levels of AsTEP1 gene is indicated by two asterisks (*).

To confirm our results, we analyzed the expression of TEP1 gene in these samples, as this gene is reported to be expressed by a type of hemocytes viz. granulocytes (Ramirez et al., 2014).

Results presented in **Figure 5.3,** revealed that the relative mRNA levels of TEP1 gene were 20-fold (p=0.0024) and ~35-fold (p=0.0023) higher in carcasses and hemocytes, respectively against sugar-fed midguts. These results confirmed that AsDBLOX is a hemocytes-specific gene and is localized in the carcasses of adult females.

5.3.3 AsDBLOX gene is induced in carcass of *P. berghei* infected mosquito

Since we did not find any induction in mRNA levels of AsDBLOX gene in 24 h post uninfected - blood fed - (BF) or *P. berghei* infected - blood fed (Inf) mosquito midguts or carcasses as compared to sugar-fed midguts or carcasses, respectively (Figure 5.1), we analysed early induction kinetics of AsDBLOX gene in *P. berghei* infection time kinetics to find out whether mosquito immunity is activated post a infectious blood meal. Hence, we analysed the expression of AsDBLOX gene at early time points in *P. berghei* infected midguts or carcasses.

We analyzed the expression of AsDBLOX gene in uninfected (controls), or *P. berghei* infected blood-fed mosquito midguts to understand the regulation of this gene during early stages (within 24 h post feeding) of malaria parasite development. Results shown in **Figure 5.4** revealed that there was a significant decrease in AsDBLOX mRNA levels in midgut at 6 h (p=0.0360) and 18 h (p=0.0410) post *P. berghei* infected blood feeding against the respective controls.

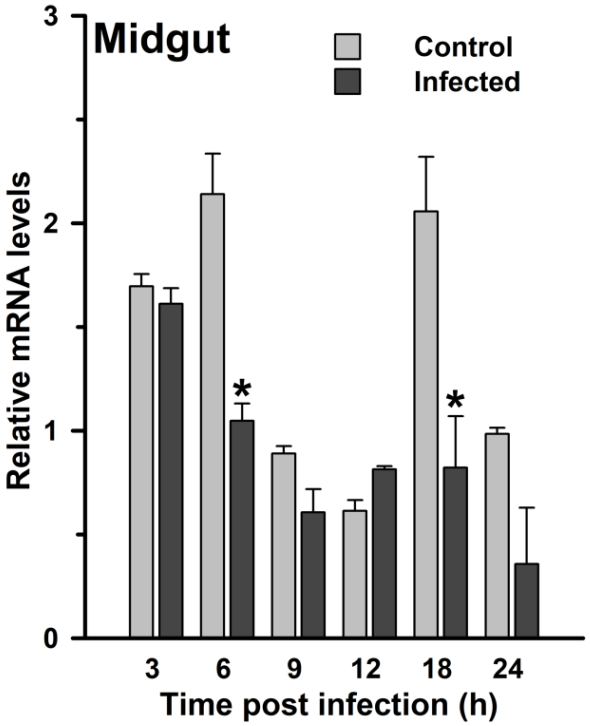


Figure 5.4: Relative expression of AsDBLOX gene in *P. berghei* infected midguts. Relative mRNA levels of AsDBLOX were analyzed in *P. berghei* infected or uninfected *An. stephensi* midguts. Significant differences $p \leq 0.05$ in relative mRNA levels of AsDBLOX gene is indicated by one asterisk (*).

The down-regulation of AsDBLOX gene in mosquito midgut tissues upon *P. berghei* infection, pointed us to further analyze the expression of AsDBLOX gene in *P. berghei* infected carcasses. The results presented in **Figure 5.5** revealed a ~2-fold induction (p=0.0411) of AsDBLOX mRNA in 3 h post *P. berghei* infected carcasses as compared to the blood-fed controls. This time point coincided with the *Plasmodium* zygote stage in the midguts of the infected females. Also, it is noteworthy to mention here that mosquitoes have an open circulatory system and hemocytes constitute major armory of mosquito innate immunity.

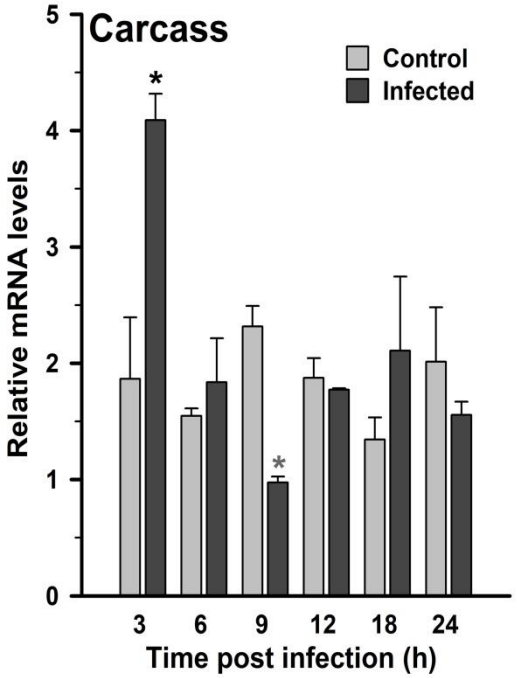


Figure 5.5: Relative expression of AsDBLOX mRNA in *P. berghei* infected carcass samples. Relative mRNA expression levels of AsDBLOX were analyzed in *P. berghei* infected *An. stephensi* midguts. Significant differences $p \leq 0.05$ in relative mRNA levels of NOS are indicated by two or one asterisks (*).

They are located in the carcass compartment of mosquito body where they move freely (motile hemocytes) or adhere to various internal organs like the abdominal wall, head or valves of the heart etc (sessile hemocytes). While infection induces an increase in the number of circulating granulocytes, sessile

hemocytes function in pathogen capture as they are localized around regions of high hemolymph flow (King and Hillyer, 2013). This meant that the expression of AsDBLOX gene in the carcass tissue is in reality, the expression in the motile hemocytes.

The induction of AsDBLOX gene in the *P. berghei* infected mosquito carcasses (**Figure 5.5**) led us to assume that this gene might have an indirect effect on *P. berghei* and for that, we analyzed the expression of *P. berghei* 28S (Pb28S) rRNA levels in infected midguts because the parasite remains in the midgut lumen or epithelium upto 24h of infection (**Figure 5.6**).The expression of Pb28S rRNA gene was found to be variable from 3 h to 24 h post infection. The significant decrease in parasite rRNA at 6 h suggested that the growth of parasite (as reflected by Pb28S rRNA levels) is negatively correlated with an induction of AsDBLOX gene. The further decrease in Pb28S rRNA at 12 h, and 18 h could be due to activation of other immune mechanisms as *Plasmodium* is reported to undergo bottleneck situations at various time points in its life cycle in the mosquito (Smith et al., 2014).

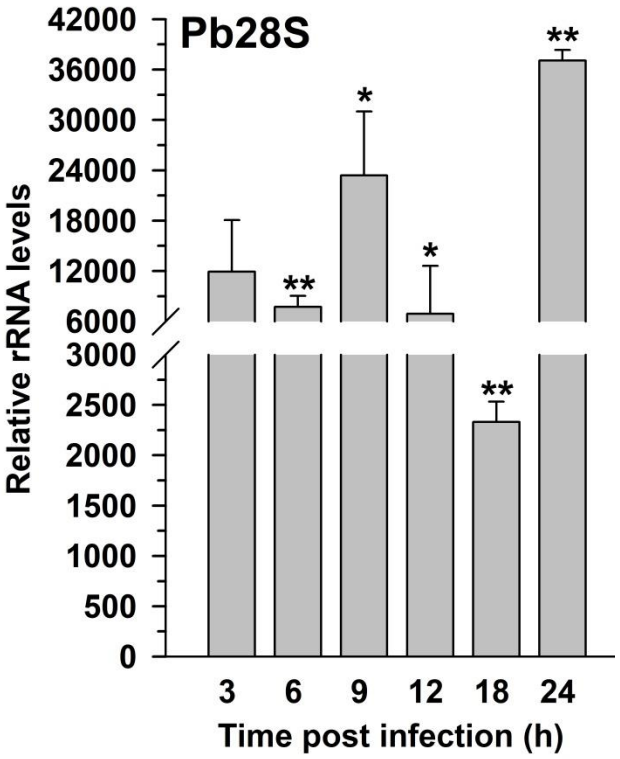


Figure 5.6: Relative levels of Pb28S rRNA in *P. berghei* infected midguts. Relative rRNA levels of Pb28S were analyzed in *P. berghei* infected *An. stephensi* midguts. Significant differences $p \leq 0.01$ or $p \leq 0.05$ in relative rRNA levels of Pb28S is indicated by two or one asterisks (*), respectively.

Moreover, as AsDBLOX is a peroxinectin, and is possibly synthesized and secreted by the hemocytes, we analyzed for the expression of TEP1 gene in *P. berghei* infected midguts. TEP1 is a carcass gene which is also synthesized and secreted by mosquito hemocytes and is known to participate in mosquito late-phase immunity (Blandin et al., 2004). It is widely reported to be involved in the killing of *Plasmodium* ookinetes, while they are traversing the midgut at ~24 h postinfection, by a TEP1-mediated lytic mechanism (de Almeida Oliveira et al., 2012).

We observed a significant ~9-fold, ~2-fold induction of TEP1 gene at 3 h (p≤0.0001) and 18 h (*p*=0.0093) post *P. berghei* infected blood meal against their respective controls (**Figure 5.7)**.

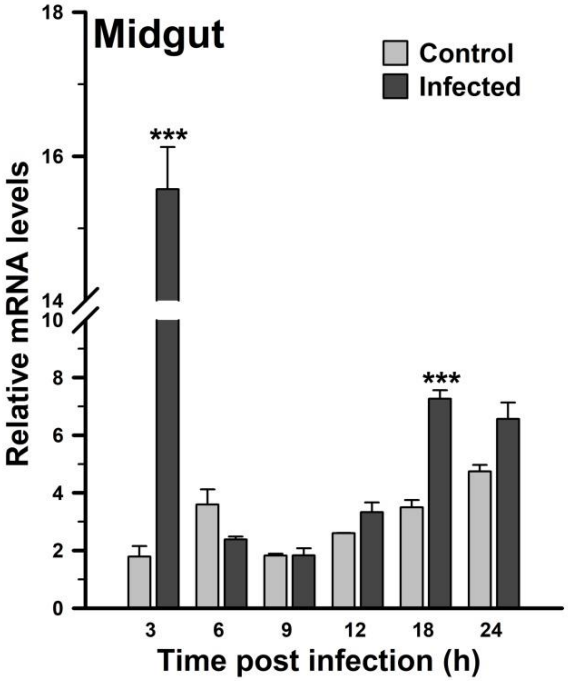


Figure 5.7: Relative expression of TEP1 gene in *P. berghei* infected midgut samples. Relative mRNA levels of TEP1 were analyzed in *P. berghei* infected *An. stephensi* midguts. Significant differences $p \leq 0.001$ or $p \leq 0.05$ in relative mRNA levels of AsTEP1 gene are indicated by two or one asterisks (*), respectively.

Its mRNA induction at an early time of blood meal indicated towards the activation of mosquito immunity at ~3 h post *P. berghei* infected blood feeding.

It is noteworthy to mention here that Pb28S rRNA expression (**Figure 5.6**) was drastically reduced at 6 h post *P. berghei* infected blood fed midguts in comparison to 3h, and we observed that TEP1 gene was induced at 3h (**Figure 5.7**). Thus, we believe that this reduction in *Plasmodium* is possibly due to hemocyte adhesion to the basal side of midgut and further induction of TEP1 gene to cause parasite killing in the midgut.

These results also correlate with previous observation by Smith et al. (2016) where they reported a dramatic shift in proteomic profiles of granulocyte populations upon phagocytosis, blood-feeding and after *Plasmodium falciparum* infection, which suggested that hemocytes have an integral role in priming the mosquito immune system against the pathogenic challenge (Smith et al., 2016).

We then analyzed the expression of NOS (nitric oxide synthase) gene in the *P. berghei* infected midguts as it was reported to be significantly induced at 6 h in *P. berghei* infected *A. stephensi* (Luckhart et al., 2003). However, we observed an induction of NOS gene by ~2-fold at 3 h (*p*=0.0482) and 24 h (*p*=0.0150) post *P. berghei* infection in *A. stephensi* midguts as compared to respective uninfected controls (**Figure 5.8**). This supported our hypothesis that mosquito hemocyte immunity is elicited against a *Plasmodium*-infected blood meal.

Moreover, mosquitoes are reported to elicit anti-plasmodial immunity against blood meal stages of the parasite (Luckhart et al., 2003; Akman-Anderson et al., 2007; Price et al., 2013). In parallel, blood feeding alone was reported to increase hemocytes proliferation in mosquitoes, and they display higher levels of critical components of the complement and melanization immune pathways 24 h post-blood meal (Bryant and Michel, 2014).

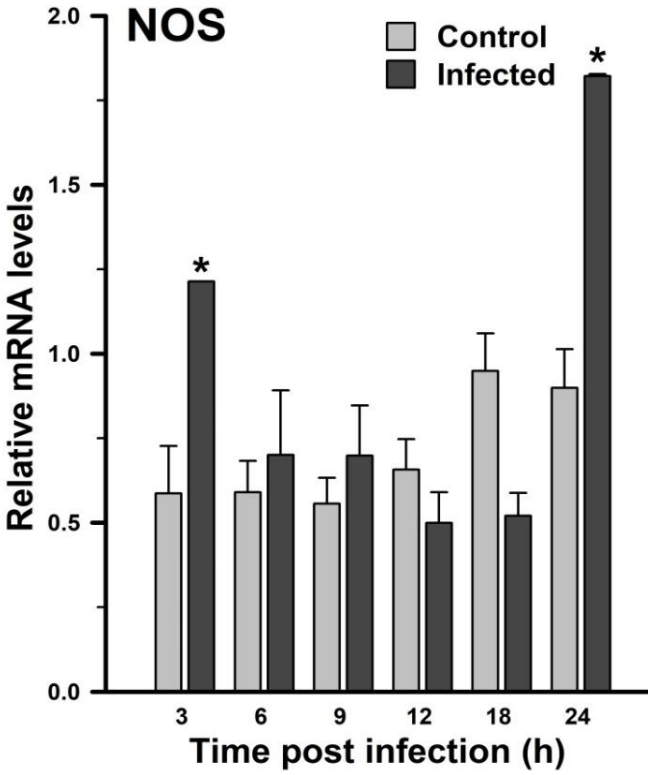


Figure 5.8: Relative expression of NOS gene in *P. berghei* infected midgut samples. Relative mRNA levels of NOS were analyzed in *P. berghei* infected *An. stephensi* midguts. Significant differences $p \leq 0.05$ in relative mRNA levels of NOS gene are indicated by two or one asterisks (*).

Thus, we propose that *An. stephensi* recognizes *Plasmodium*-infected blood meal in the early stages of infection and induces NOS gene. The NOS catalyzed molecules in turn trigger hemocytes adhesion to the basal lamina at ~3 h (possibly mediated by AsDBLOX) and kills *Plasmodium* in the midgut through induction of TEP1. This thus explains the reduction in parasite numbers (indicated by reduced Pb28S rRNA gene) in the infected midguts at 6 h post infection.

5.3.4 Bacterial challenge induces AsDBLOX gene in larvae

Based on our previous results, AsDBLOX is a peroxinectin and is synthesized and secreted by the hemocytes which are reported to participate in antibacterial immunity of mosquitoes. Hence, we were interested in understanding the regulation of AsDBLOX gene in anti-bacterial immune responses of mosquitoes. For this, we challenged *An. stephensi* larvae with *E. coli* and *M. luteus*. The successful bacterial infection of larvae was evaluated by studying the 16S rRNA (bacterial gene) expression in the challenged samples against the uninjected controls. Results presented in **Figure 5.9** revealed that

16S rRNA was 30-fold at 1 h, 150-fold at 2 h and 400-fold at 12 h post injection, respectively against their respective sham-treated controls. It is noteworthy to mention that larvae being aquatic in nature are continuously exposed to bacteria and mount a strong anti-bacterial response in comparison to the aerial habitat of adult mosquitoes (League et al., 2017). This pattern of bacterial proliferation coincides with a previous report of infection in insects by *Xenorhabdus nematophila* bacteria as reported by Sicard et al. (2004).

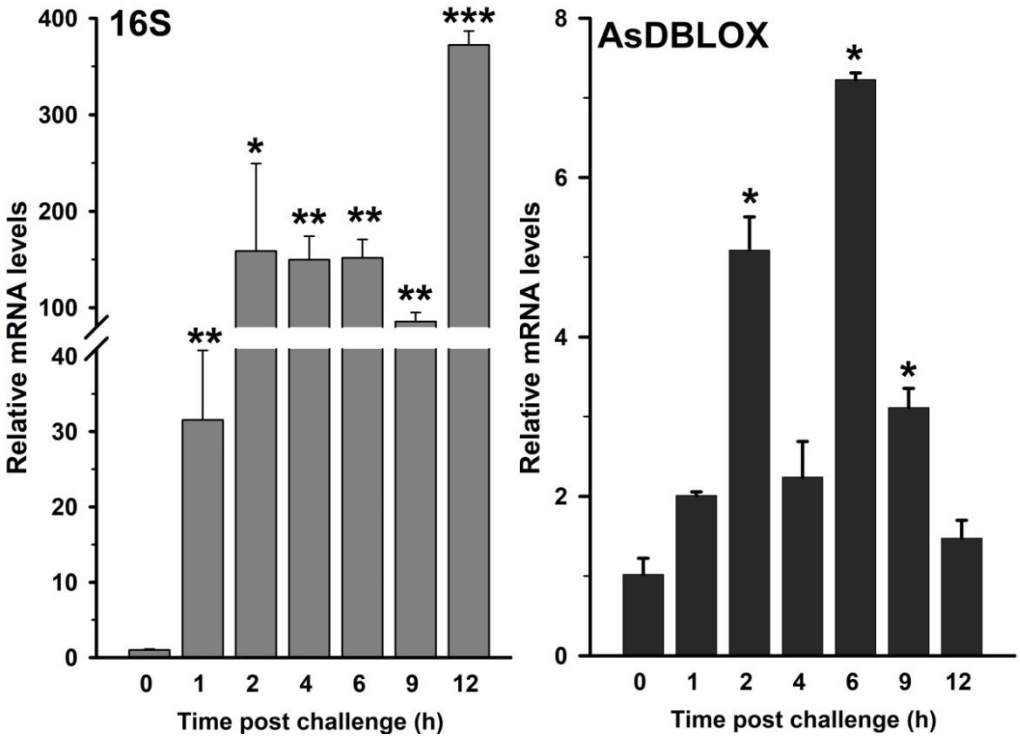


Figure 5.9: Relative expression of 16S rRNA and AsDBLOX mRNA in bacteria challenged mosquito larvae Relative rRNA and mRNA expression levels of 16S and AsDBLOX, respectively, were analyzed in bacteria challenged *A. stephensi* larvae. Significant differences $p \leq 0.0001$ or $p \leq 0.001$ $p \leq 0.05$ in relative rRNA levels of 16S and mRNA levels of AsDBLOX are indicated by three or two or one asterisks (*),

Further, we analyzed the expression of AsDBLOX gene in the bacteria challenged larvae and observed that the gene was significantly induced by ~5-fold at 2 h (p=0.0198) and ~7-fold at 6 h (p=0.0112) against their respective sham-treated controls, thus displaying a biphasic pattern of induction. The mRNA expression of AsDBLOX gene was significantly down-regulated at 9 h (p=0.0369) against the 0h control.

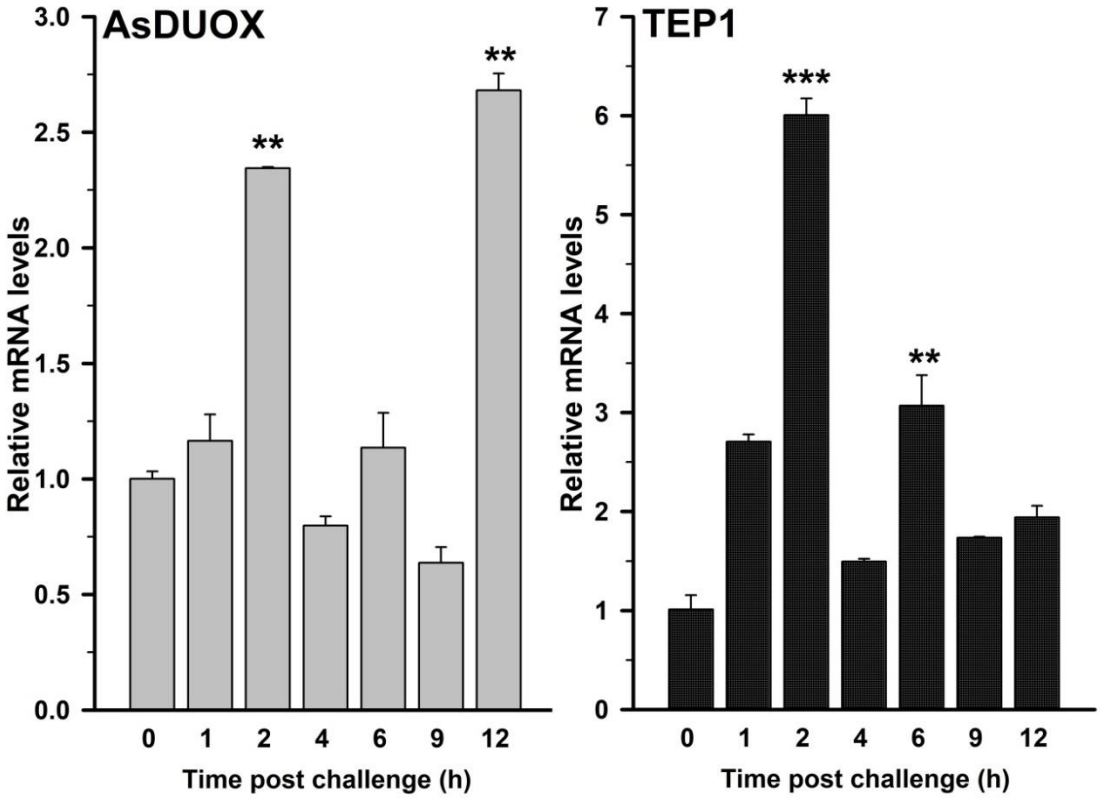


Figure 5.10: Relative expression of AsDUOX and TEP1 gene in bacteria challenged mosquito larvae. Relative mRNA levels of AsDUOX and TEP1 were analyzed in bacteria challenged *A. stephensi* larvae. Significant differences $p \leq 0.001$ or $p \leq 0.01$ in relative mRNA levels of AsDUOX and TEP1 are indicated by three or two asterisks (*), respectively.

The induction of AsDBLOX gene at 2 h coincided with the induction of 16S rRNA, and its induction at 6 h could be due to exhaustion of AsDBLOX transcripts which caused transcriptional up-regulation. Also, we observed that both AsDBLOX gene and 16S rRNA were downregulated at 9 h post infection as compared to the control. Thus the induction of AsDBLOX gene post bacteria injection suggests the activation of mosquito humoral immunity.

We further analyzed the expression of another heme peroxidase- *An. stephensi* double oxidase – AsDUOX in bacteria-challenged larvae, as DUOX enzyme is responsible for ROS generation that then act as substrates for other HPXs (Hurd et al., 2015). Results presented in **Figure 5.10** showed that AsDUOX gene was induced at 2 h (p=0.0013) and 12 h (p=0.003) against the sham-treated controls, which coincided with the induced expression of 16S rRNA at the same time points. This showed that AsDUOX gene displays a biphasic pattern of induction in a way similar to the AsDBLOX

gene that reveals the involvement of two heme peroxidases in mosquito immunity against the bacterial challenge.

These results prompted us to explore the anti-bacterial immune responses of *An. stephensi* larvae further. Firstly, we determined for the expression of TEP1 gene in the bacteria challenged larvae. Previously the TEP1 was reported to be strongly up-regulated after bacterial challenge in *Drosophila* and *An. gambiae* (Blandin et al., 2004; Levashina et al., 2001; Lagueux et al., 2000).

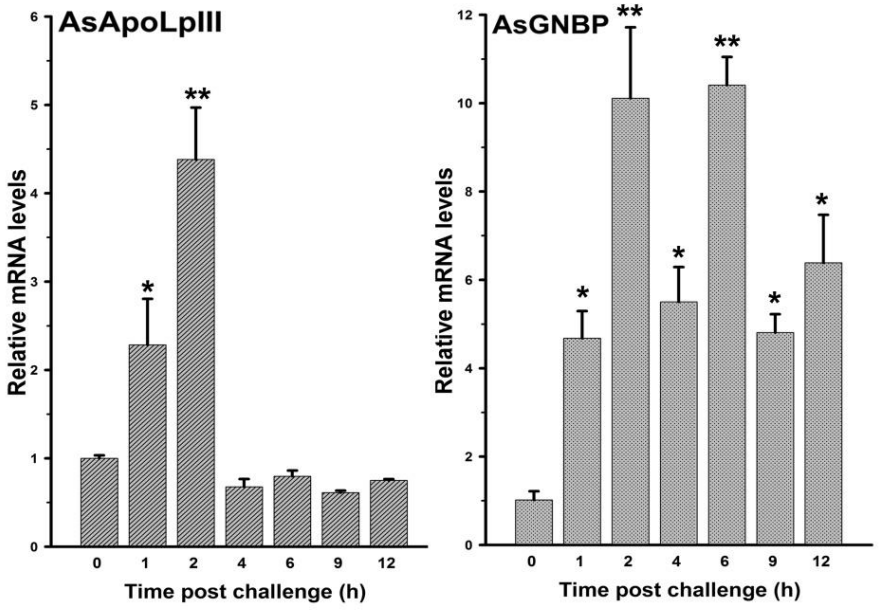


Figure 5.11: Relative expression of AsApoLPIII and GNBP genes in bacteria challenged mosquito larvae. Relative mRNA levels of AsApoLPIII and GNBP were analyzed in bacteria challenged *A. stephensi* larvae. Significant $p \leq 0.001$ or $p \leq 0.01$ *or* $p \leq 0.05$ in relative mRNA levels of AsApoLPIII and GNBP genes are indicated by two or one asterisks (*), respectively.

Results presented in **Figure 5.10** showed that TEP1 was significantly induced by 2.5-fold, 6-fold, and 3-fold at 1 h (p=0.004), 2 h (p≤0.001) and 6 h (p≤0.01), respectively in the bacteria challenged larvae against their sham-treated controls. The 16S rRNA gene was also induced at 2 h post challenge and this correlated with the increase in mRNA expression of AsDBLOX and TEP1 genes (**Figure 6.9, 6.10**). These findings suggested the activation of an effective antibacterial immune response in larvae.

Subsequently, we analyzed the expression of other immune genes widely reported to be elicited against bacterial infection in insects – Apolipophorin III, GNBP and transcription factor REL1 (Whitten et al., 2004; Zdybicka-Barabas et al., 2015; Dimopoulos et al., 1997; Gross et al., 1999).

Apolipophorin III (ApoLPIII) is a hemolymph protein which has a functional role in both lipid transport and immune responses of insects. We analyzed its expression in

bacteria-challenged larvae and observed that ApoLPIII was significantly induced by ~2-fold (p=0.037) and ~4.5-fold (p=0.0079) at 1 h and 2 h post bacterial challenge as compared to the sham-treated controls (**Figure 5.11**). This further strengthened our hypothesis that the increase in proliferation of bacteria at 2 h strongly activated various immune molecules to combat the infection.

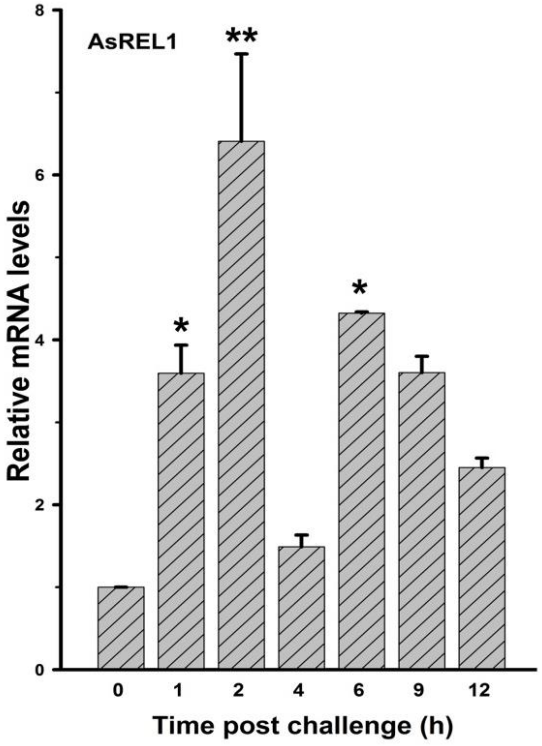


Figure 5.12: Relative expression of Rel1 gene in bacteria challenged mosquito larvae. Relative mRNA levels of Rel1 gene were analyzed in bacteria challenged *A. stephensi* larvae. Significant $p \leq 0.001$ or $p \leq 0.01$ *or* $p \leq 0.05$ in relative mRNA levels of Rel1 gene are indicated by three or two or one asterisks (*), respectively.

The anti-plasmodial and antibacterial defenses in *Anopheles* are mainly controlled by the Toll and Imd immune signaling pathways (Clayton et al., 2014). While the Toll pathway is primarily elicited by Gram-positive (G+) bacteria, fungi, viruses and *Plasmodium* (Ramirez and Dimopoulos, 2010), the Imd pathway is induced by Gram-negative (G−) and Gram-positive (G+) bacteria and *Plasmodium* (Meister et al., 2005; Cirimotich et al., 2010; Clayton et al., 2013; Clayton et al., 2014). GNBP (Gram-negative binding protein), is a pattern recognition receptor (PRR) of Toll pathway and is reported to be elicited upon bacterial infection in mosquitoes (Dimopoulos et al., 1997). Hence, we evaluated the regulation of this gene upon bacterial challenge to mosquito larvae (**Figure 5.11**). We observed that it was induced by 5-fold at 1 h (p=0.024) which increased to 10-fold at 2 h (p=0.004) and 6h (p=0.005), and 6-fold at 12 h (p=0.044) against the sham-treated controls.

Also, the activation of Toll and Imd pathways via the recognition of PAMPs (Pathogen Associated Molecular Patterns) ultimately leads to the nuclear translocation of the NF-κB transcription factors REL1 and REL2, respectively. REL1 is a transcription factor and the homolog of *Drosophila* Dorsal. It is reported to be elevated post bacterial challenge in larvae and adults (Gross et al., 1999). The induction of REL1 mRNA levels by 4-fold, 6-fold, and 1-fold at 1 h (p≤0.05), 2 h (p≤0.01) and 6 h (p≤0.05) post-infection,

respectively against the controls suggested the activation of Toll pathway (**Figure 5.12**). Hence, bacterial challenge in larvae activates the hemocytes which finally lead to the elicitation of an array of immune genes to combat the microbial population.

5.3.5 *An. stephensi* females combat adult bacterial infection by a possible interplay of redox homeostasis and immune activation

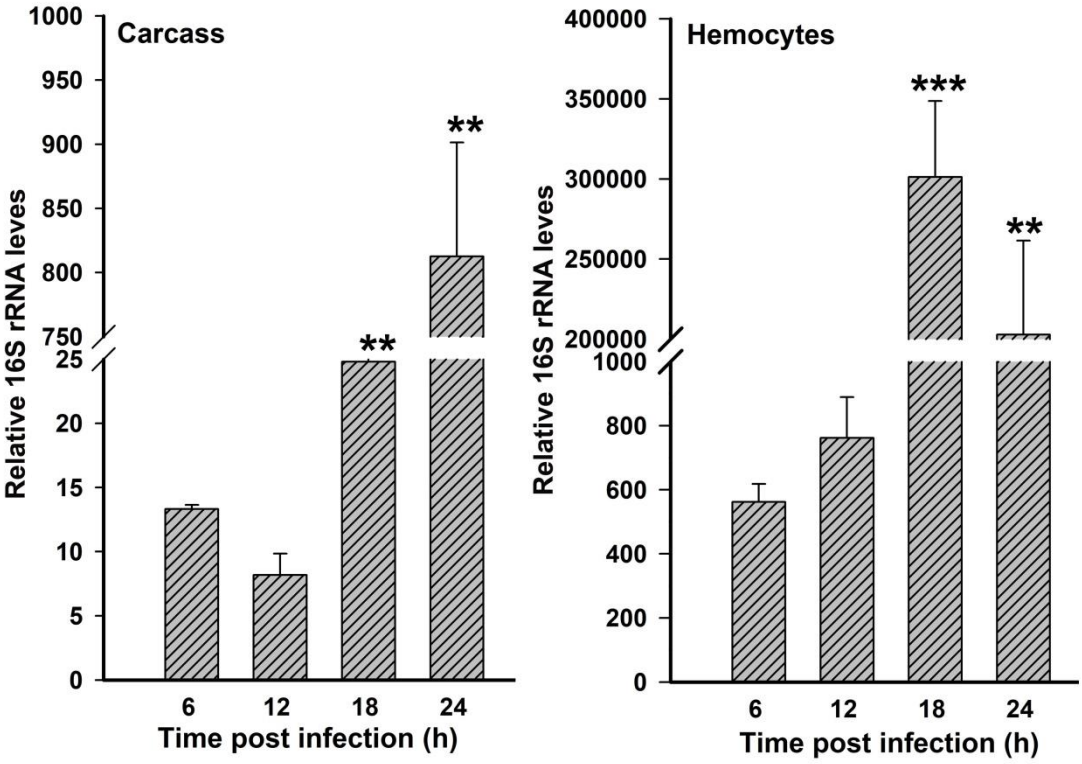


Figure 5.13: Relative expression of 16S rRNA in bacteria challenged mosquito adult. Relative rRNA expression levels of 16S were analyzed in bacteria challenged *A. stephensi* adult carcasses and hemocytes. Significant differences $p \leq 0.001$ in relative rRNA levels of 16S is indicated by two asterisks (*).

Our next approach was to investigate the regulation of AsDBLOX gene in hemocytes from bacteria challenged adult mosquitoes. Briefly, we challenged adult female mosquitoes with *E. coli* and *M. luteus* bacteria and collected carcasses and hemocytes at different time points to analyze the transcriptional regulation of various genes. The infection of bacteria was examined through 16S rRNA levels in the infected samples against their controls. Results presented in **Figure 5.13** showed a 15 fold 16S rRNA level at 6 h ($p \leq 0.01$) post injection as compared to controls, which indicated a bacterial infection. These rRNA levels significantly reduced by ~7-fold at 12 h and then increased by ~25-fold and ~800-fold at 18 h and 24 h, respectively against the sham-treated controls.

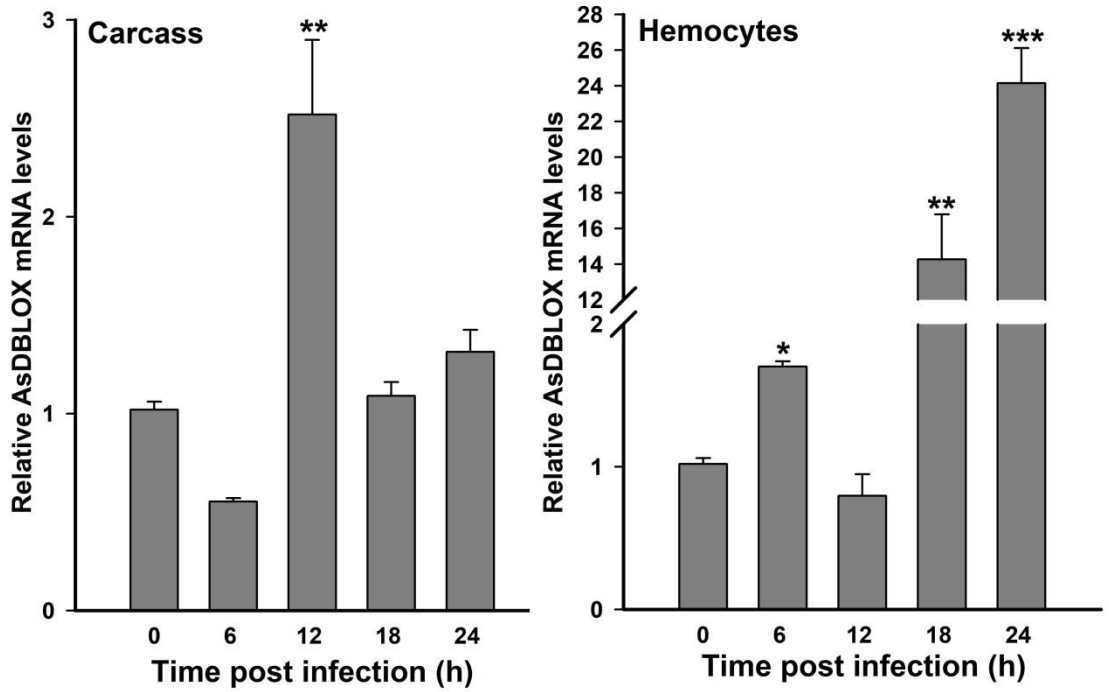


Figure 5.14: Relative expression of AsDBLOX mRNA in bacteria challenged mosquito adult. Relative mRNA expression levels of AsDBLOX were analyzed in bacteria challenged *A. stephensi* adult carcasses and hemocytes. Significant differences $p \leq 0.0001$, $p \leq 0.001$, $p \leq 0.05$ in relative mRNA levels of AsDBLOX gene are indicated by three, two or one asterisks (*).

A similar pattern of induction in 16S rRNA levels was observed in the hemocytes, with 550-fold and 750-fold induction at 6 h and 12 h, respectively against the controls. Further, the 16S rRNA increased exponentially to ~3 X 10^5 fold and ~2 X 10^5 fold at 18 h and 24 h, respectively, against their controls.

Further, peroxinectins are reported as cell adhesion heme peroxidases involved in innate immunity, we analyzed the relative mRNA level of AsDBLOX gene in these bacteria challenged adult mosquitoes (**Figure 5.14**). We observed that there was a significant ~3-fold induction of AsDBLOX gene in bacteria-challenged mosquito carcasses at 12 h, while in the hemocytes the gene was induced by 2 fold ($p \leq 0.05$), 14-fold ($p = 0.0028$) and ~24-fold ($p \leq 0.001$) at 6h, 18 h and 24 h respectively, against the sham-treated controls. This pattern of induction was concomitant with the 16S rRNA expression levels at 18 h and 24 h observed in **Figure 5.13**.

Further, we investigated the mRNA levels of TEP1 gene in these bacteria challenged samples. Results presented in **Figure 5.15** showed that TEP1 gene was induced in the carcasses post bacterial challenge against their controls, while in the hemocytes, the gene was significantly up-regulated by 11-fold at 18 h ($p \leq 0.001$) as

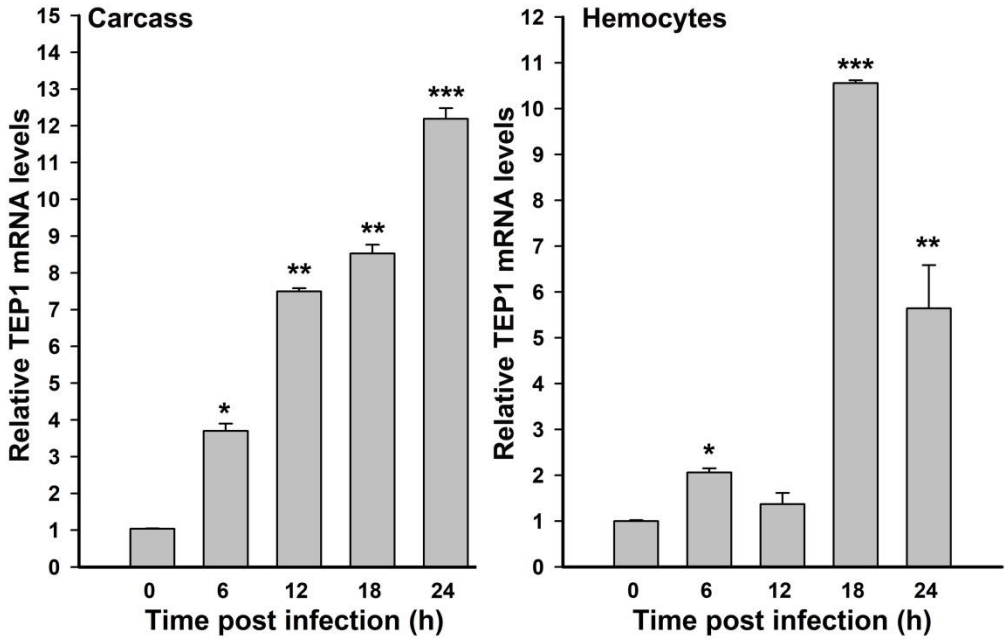


Figure 5.15: Relative expression of TEP1 in bacteria challenged mosquito adult. Relative mRNA expression levels of TEP1 were analyzed in bacteria challenged *A. stephensi* adult carcasses and hemocytes. Significant $p \leq 0.001$ or $p \leq 0.01$ *or* $p \leq 0.05$ in relative mRNA levels of TEP1 are indicated by three or two or one asterisks (*), respectively.

compared to the control. These results indicated that bacteria overproliferated post 18 h of their inoculation into the mosquito and induced AsDBLOX and TEP1 genes as evident by the 16S rRNA levels at the same time. Hence, these results demonstrated that AsDBLOX gene might be possibly acting as a peroxinectin having an anti-bacterial role in carcass and hemolymph immunity.

Further, we examined the mRNA levels of EC-SOD, an antioxidative enzyme. It is an extracellular SOD which was reported previously to show maximum expression in unchallenged *Tribolium castaneum* insect and was significantly down-regulated upon bacterial challenge (Ferro et al., 2017). This could be because SODs belong to the family of antioxidative enzymes which balance the homeostasis of oxidative stress in both vertebrates and invertebrates. Bacterial injection into insects increases oxidative stress in the hemolymph as has been reported previously in larvae of silkworm *Bombyx mori* (Ishii et al., 2008). The increased ROS levels eliminate pathogens and parasites in

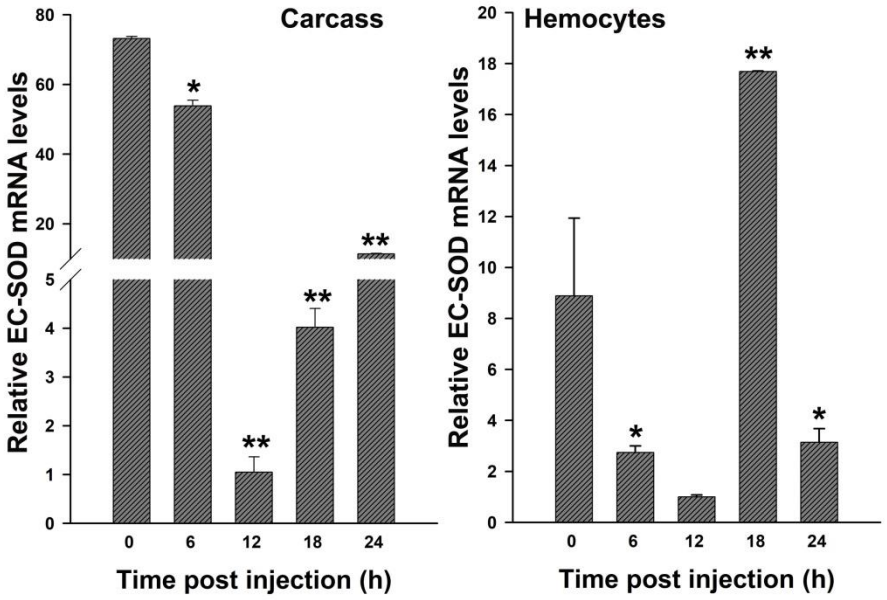


Figure 5.16: Relative expression of EC-SOD in bacteria challenged mosquito adult. Relative mRNA expression levels of EC-SOD were analyzed in bacteria challenged *An. stephensi* adult carcasses and hemolymph. Significant $p \leq 0.01$ *or* $p \leq 0.05$ in relative mRNA levels of EC-SOD are indicated by two or one asterisks (*), respectively.

insects (Nappi and Ottaviani, 2000). The decrease of EC-SOD gene expression in the carcasses and hemocytes might be supporting an increase in oxidative stress in mosquito body to kill the injected bacteria. After the bacterial population was significantly reduced, the enzyme transcription was reinitiated at later time points (**Figure 5.16**). Another possible explanation is that EC-SOD transcripts were down-regulated upon bacterial challenge due to rapid translation for balancing the high oxidative stress, and the transcription reinitiated upon possible exhaustion of transcripts post 18 h of challenge. These results are in agreement with a previous report by Jiang et al. (2018), where EC-SOD gene was down-regulated in sea cucumber *Apostichopus japonicas* after 4 h of bacterial challenge.

5.3.6 AsDBLOX gene silencing increases bacterial load

To further validate our hypothesis that AsDBLOX is a crucial molecule of mosquito innate immunity, we used gene silencing approach. Briefly, LacZ or AsDBLOX dsRNA was injected into newly emerged sugar-fed females, and they were then challenged with a mixture of *E. coli* and *M. luteus* bacteria as discussed in Materials and Methods.

AsDBLOX silencing was confirmed at 18 h (p=0.040), and 24 h (p=0.0032) post bacterial challenge in hemocytes in the silenced group of mosquitoes against the LacZ injected control mosquitoes (**Figure 5.17**).

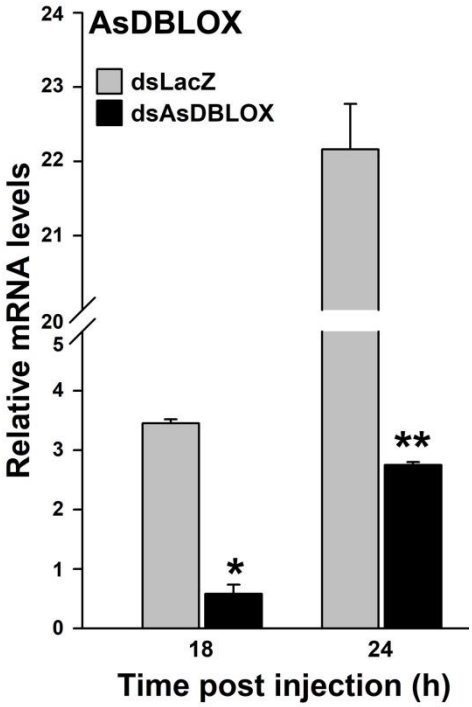


Figure 5.17: AsDBLOX silencing in hemocytes of bacteria challenged *Anopheles* stephensi mosquitoes Relative mRNA abundance of AsDBLOX gene in hemocytes at 18 h and 24 h post bacteria challenged mosquitoes injected with dsLacZ or dsAsDBLOX. Significant differences $p \leq 0.001$ or $p \leq 0.05$ in relative mRNA levels of AsDBLOX are indicated by two or one asterisks (*), respectively.

Then, we examined the levels of 16S rRNA in these samples, to analyze the effect of AsDBLOX silencing on bacterial infection (**Figure 5.18).** We observed that upon AsDBLOX gene silencing, there was a ~800- fold and ~1500-fold induction in 16S rRNA levels at 18 h and 24 h respectively, against the dsLacZ controls. This indicated a crucial role of AsDBLOX in controlling bacterial population.

Further, we analyzed TEP1 gene expression in the silenced hemocytes, and we found that this gene was significantly induced at 18 h ($p \leq 0.05$) and 24 h ($p \leq 0.01$) post bacterial challenge. This indicated activation of mosquito humoral

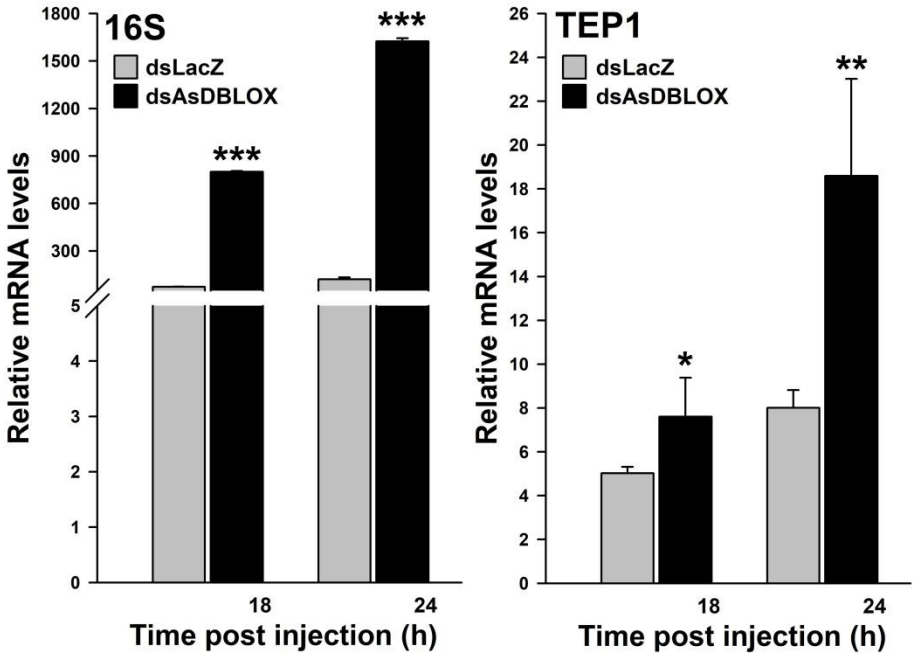


Figure 5.18: Effect of AsDBLOX silencing in bacteria challenged mosquito adult on 16S rRNA and TEP1 gene. Relative expression of 16S rRNA and TEP1 mRNA in 18 h and 24 h post bacteria challenged mosquitoes injected with dsLacZ or dsAsDBLOX. Significant differences $p \leq 0.0001$ or $p \leq 0.001$ or $p \leq 0.05$ in relative rRNA levels of 16S and mRNA levels of TEP1 are indicated by three or

immunity upon AsDBLOX silencing (**Figure 5.18**). AsDBLOX, a peroxinectin, is an adhesion molecule synthesized by hemocytes. TEP1 is reported to kill pathogens by a complement like lytic mechanism. Hence, based on our results, we propose that AsDBLOX possibly participates in hemocytes antibacterial response by a cell-adhesion mechanism.

5.4 Discussion

In insects, peroxinectins are the peroxidases that help in the adhesion of hemocytes to the extracellular matrix using their integrin binding motifs. AsDBLOX is a type of peroxinectin that has two peroxidase domains – HPX domain I and II. While the HPX domain I has two integrin binding motifs, the HPX domain II possibly functions as a heme peroxidase (**Chapter 3** and **4**). This finding is different from previous reports of peroxinectins which have only one HPX domain and one integrin binding motif (Albelda and Buck, 1990; Johansson et al., 1995; Liu et al., 2004a; Kajla et al., 2015). This gene structure was unique and hence directed us to perform a functional characterization of AsDBLOX in *An. stephensi.*

In our initial gene expression studies, we found that AsDBLOX was not induced in midgut or carcass tissue at 24 h post uninfected - or *P. berghei* infected - blood feeding. Therefore, AsDBLOX is not a blood-induced gene. In contrast, this gene showed maximum mRNA expression in sugar-fed mosquitoes. This led us to hypothesize that this gene might be playing a functional role in carcass compartment or more specifically the hemocytes, as it was predicted to be a secretory protein and peroxinectin are reported to be synthesized and secreted by the granulocytes (Liu et al., 2004a). Hence, we proceeded towards tissue-specific localization of AsDBLOX gene and collected hemocytes from sugar-fed mosquitoes. The results revealed that DBLOX is a hemocytes specific gene.

Next, we analyzed the regulation of AsDBLOX gene in mosquitoes challenged with bacteria. We observed a significant induction of this gene in both larvae, and adult mosquitoes post bacterial challenge. Our hypothesis was further strengthened by the RNAi silencing approach, where we found that the silenced mosquitoes had increased 16S rRNA levels against the non-injected controls, indicative of the bacterial population. Moreover, we observed that the relative mRNA levels of TEP1 gene (a hemocyte-specific gene) was induced in these bacteria challenged larvae and adults. Thus, both *An. stephensi* larvae and adults mount a strong anti-bacterial response.

Hemocyte encapsulation response has been widely studied in various insects, but how do they adhere to each other and form a capsule around the periphery of the pathogen has not been well understood. Since peroxinectin are implicated in cell adhesion and AsDBLOX has two integrin binding motifs, based on our results, we believe that AsDBLOX mediates hemocyte adhesion and also acts as a heme peroxidase.

5.5 Conclusion

AsDBLOX is a unique heme peroxidase that has two peroxidase domains. It also has integrin binding motifs which facilitate the binding of the AsDBLOX protein to other hemocytes and other cells (fat body, organs) in the mosquito carcass.

The challenge of bacteria through cuticle puncture elicits a whole spectrum of repair mechanisms alongside combating the pathogen stress. A whole array of antioxidative machinery comes into play, and it causes the down-regulation of antioxidant enzyme SOD so that ROS can mediate the killing of the pathogen. On the other hand, hemocytes recognition of the pathogens by the PRRs triggers the process of hemocytes encapsulation leading to activation of prophenoloxidase cascade which will cause melanization and eventual killing of the bacteria. The up-regulation of TEP1, a hallmark molecule of the humoral immune system is also triggered, for the successful clearance of the pathogen. Thus this is a classic example of the coordinated action of humoral and cellular branches of immunity.

Chapter 6

Identification and Characterization of an *Anopheles* heme peroxidase - AsHPX8

6.1 Abstract

In this chapter, we carried out the molecular characterization of heme peroxidase HPX8 from Indian malaria vector *An. stephensi*. It encodes for a peroxinectin-like secretory protein of 770 amino acids. Phylogenetic analysis of AsHPX8 with heme peroxidases from various other organisms revealed that its orthologs are present only in Phylum Arthropoda, which means that HPX8 is an Arthropod-specific gene. Although the gene is expressed in all the developmental stages of *An. stephensi*, it showed maximum expression in adult male mosquitoes; thus AsHPX8 is a gender-biased gene. However, in adult female mosquitoes, the gene was induced in both midguts and carcasses post blood feeding. Also, the gene was induced in blood/saline supplemented with bacteria fed midguts, which indicated that the gene is induced upon bacterial growth. Moreover, we observed that AsHPX8 gene was induced in adult carcasses post blood feeding, and its gene silencing induced the expression of its duplicated paralog - AsHPX7. Thus, AsHPX8 is an arthropod-specific gene which plays a crucial role in mosquito physiology as well as immunity and thus can be exploited to control vector population in malaria-endemic regions.

6.2 Introduction

Plasmodium undergoes bottleneck situation several times in the course of its life cycle, and the first mosquito organ it interacts with is the mosquito midgut (Sinden and Billingsley, 2001; Ghosh et al., 2000; Pradel, 2007). The midgut harbors a variety of bacteria which live in a symbiotic relationship with the mosquito (Minard et al., 2013; Kajla et al., 2015a). These bacteria produce various enzymes and toxins whereas the mosquito induces reactive oxygen species (ROS) upon bacterial proliferation (Cirimotich et al., 2011). These ROS and toxins are harmful to *Plasmodium* (Smith et al., 2014). Thus, exploring key immune molecules involved in maintaining gut microbiota can be used as an excellent tool to develop *Plasmodium* control strategies (Dong et al., 2009; Cirimotich et al., 2011).

Kumar et al. (2004) reported the induction of five HPX genes in the *An. gambiae* midgut in response to *Plasmodium* infection – HPX8, HPX2, HPX7, DBLOX, and DUOX. Also, AgHPX8 gene was reported to be induced in *Plasmodium*-infected *An. gambiae* midguts upon silencing of another heme peroxidase - AgHPX15 (HPX15 forms a mucin barrier that blocks the interaction of blood bolus bacteria with the immune reactive midgut epithelium). However, AgHPX8 gene was not induced when gut bacteria were

removed with antibiotics (Kumar et al., 2010a). Thus, AgHPX8 functions as a crucial molecule in the midgut of *An*. *gambiae*. Moreover, AgHPX8 is an ortholog of the *D. melanogaster* peroxinectin (*Dpxt*). *Dpxt* is a peroxinectin gene preferentially expressed during oogenesis and early embryogenesis and AgHPX8 is also reported to be involved in oogenesis (Vazquez et al., 2002; Emami et al., 2017). Also, in *Aedes aegypti*, chorion peroxidase- AAEL004386 gene was induced following a blood meal (Amenya et al., 2010).

Insects eggs are characterized by hardening and melanization of chorion or eggshell (produced by the follicle cells) which prevents their desiccation. This hardening of egg chorion is mediated by a) peroxidase-mediated chorion protein crosslinking through dityrosine formation and b) phenol oxidase-catalyzed chorion melanization in *Ae. aegypti* (Li et al., 1996). Also, in *Aedes aegypti*, chorion peroxidase- AAEL004386 gene was induced following a blood meal . Hence, HPX8 gene possibly also plays a crucial role in insect reproductive process.

These facts prompted us to perform a molecular and functional characterization of HPX8 gene in *An. stephensi* and analyze its transcriptomic profile in mosquito midgut upon blood feeding and bacterial infection. We also analyzed its possible role as a chorion peroxidase in the *An. stephensi* eggshell (chorion). Elucidation of its exact function can prove it as a valuable target for controlling the mosquito population.

6.3 Results

6.3.1 PCR amplification, full-length sequencing and characterization of AsHPX8 gene from *An. stephensi*

To identify AsHPX8 gene in the genome of *An. stephensi,* PCR-based approach was used. AgHPX8 gene (AGAP004038) of 2515 bp length was BLAST searched against *An. stephensi* genome available at NCBI, and contig 4435 (match between 48266124 and 48268778 nucleotides) (SuperContig KB664356, Ensembl identifier ASTE008825 in the annotated genome of *An. stephensi*) with a query coverage of 99%, 78% identity and E value 0 was selected. Gene-specific primers were designed as described in the **Materials and Methods** (**Section 2.3**). *An. stephensi* genomic DNA (gDNA) or cDNA (midgut or carcass) were used as templates to amplify AsHPX8 gene. **Figure 6.1A** shows that we did not obtain desired bands with F1-R1 primers; however, a single band corresponding to ~500bp was obtained using F2-R2 primers (further used for qPCR

studies). Also, we obtained single desirable band with primers F3-R3 (used for dsRNA synthesis) (**Figure 6.1B)**.

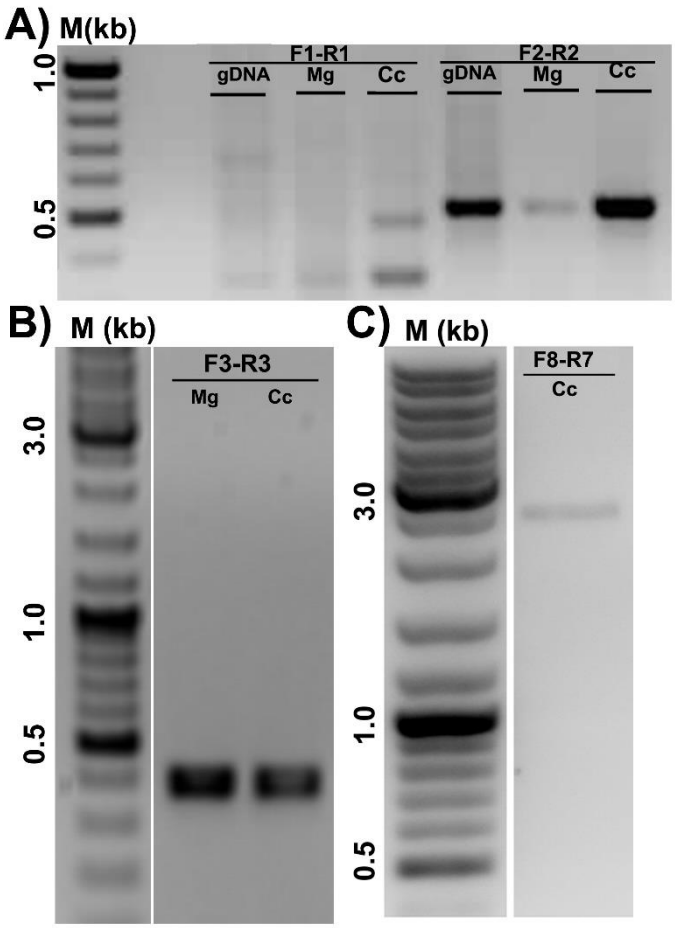


Figure 6.1: PCR amplification of *An. stephensi* AsHPX8 gene. A. *An. stephensi* genomic DNA (gDNA), 24h post blood fed midgut (Mg) or carcass (Cc) cDNA template was PCR amplified using F1-R1 (~500bp) and F2-R2 (~500bp) primers **B**. PCR amplification by primers F3-R3 (~400bp) **C**. PCR amplification of full length AsHPX8 gene from carcass (Cc) using F8-R7 primers (3kb). The left lane (M) represents the DNA ladder SM0331 used as reference for identifying the product size.

6.3.2 AsHPX8 shares significant similarity with AgHPX8

From the *An. stephensi* contig 4435, the full-length putative AsHPX8 gene was predicted using GENSCAN web server. The predicted AsHPX8 gene is 2706 bp long. It contains an open reading frame (ORF) of 2313 bp that encodes a 692 amino acid long protein. The predicted 5′-untranslated region (5´- UTR) is 116 bp, and the 3′-untranslated region is 227 bp (3´- UTR). The signal for polyadenylation TTTTTAAAAA was found at the 194 bp downstream from stop codon in 3´- UTR **(Figure 6.2)**. Further, primers for full-length PCR amplification were designed based on the alignment between AgHPX8 and AsHPX8 gene sequences as mentioned in **Materials and Methods** (**Table 2.2**) and PCR product was amplified using cDNA of 24 h blood fed carcass as a template. The F8-R7 primers amplified the expected PCR product of ~3 kb (**Figure 6.1C**). The product was sequenced, and the final consensus sequence of 2313

bp was blasted against the NCBI nucleotide database. It showed 84% identity with its ortholog AgHPX8 and was submitted to the NCBI (GenBank database accession number: KY363391). Further, the AsHPX8 gene was aligned with the *An. stephensi* genomic sequence (contig 4435), to determine the exon-intron boundaries and we

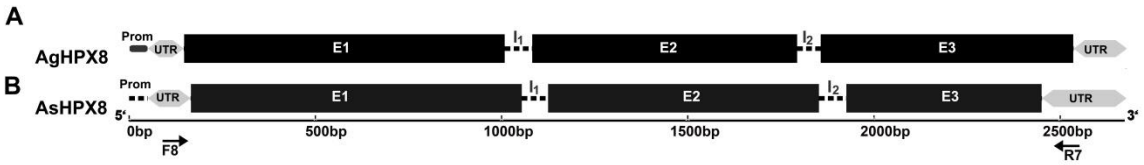


Figure 6.2: Genomic organization of HPX8 gene. A) AgHPX8 gene CDS sequence available on NCBI was used to find exonic, intronic, 5´ and 3´ UTR regions. It revealed the presence of three exons viz. E1 882 bp, E2 728 bp, E3 694 bp (total CDS reported: 2515 bp) and two introns I1 75 bp and I2 65 bp respectively. The gene promoter (Prom) of 50 bp was predicted 100 bp 5' upstream (5´ UTR) to the protein initiation codon while 3´ UTR was of 156 bp. B) The predicted AsHPX8 gene has three exons viz. E1 889 bp, E2 728 bp and E3 526 bp (total CDS 2313 bp) and two introns I1 70 bp and I2 72 bp, respectively. The gene promoter (Prom) of 50 bp was predicted 116 bp 5´ upstream (5'UTR) to the protein initiation codon while 3´ UTR was of 227 bp. Numbering indicates the nucleotide position of the orthologs. The positions of forward (F8) and reverse (R7) primers used for full-length gene sequencing are depicted as mentioned in Table 1).

found that AsHPX8 gene has three exons viz. E1 (889bp), E2 (728bp) and E3 (526bp) and two introns I1 (70bp) and I2 (72bp). Sequence comparison with its ortholog - AgHPX8 revealed that AsHPX8 had similar gene structure (**Figure 3.3**) with three exons viz. E1 (882bp), E2 (728bp) and E3 (694bp) (total CDS reported: 2515 bp) and two introns I1 (75bp) and I2 (65bp), respectively. The predicted 5´- UTR and 3´- UTR were 166 bp and 227 bp, respectively. Also, the nucleotide BLAST of AsHPX8 gene revealed first hit as AgHPX8 with 95% query coverage, 78% identity and E value 0.0. Thus, these two genes share similar exonic and intronic organization which suggested that they are true orthologs of each other. Further analysis of AsHPX8 and AgHPX8 cDNA, revealed that AsHPX8 (770aa) encodes for a protein that is three amino acids longer than AgHPX8 (767aa).

6.3.3 Conserved domain analysis of AsHPX84

Based on the conserved regions of AgHPX8 (AGAP004038), we analyzed the structure of AsHPX8 gene. The AgHPX8 gene encodes for a protein of 767 amino acid residues having molecular weight 161.7626 kDa and theoretical pI of 6.32 while AsHPX8 also had a similar architecture with 770 amino acid residues (Molecular weight: 163.4782 g/mol and theoretical pI of 6.6458 (**Figure 6.2**). The AsHPX8 protein was further

analyzed using CDD (Conserved Domain Database, NCBI) to predict conserved domains in this protein.

Results shown in **Figure 6.3** revealed that AsHPX8 shared characteristic binding sites such as homodimer interface, heme, and calcium binding sites with its ortholog

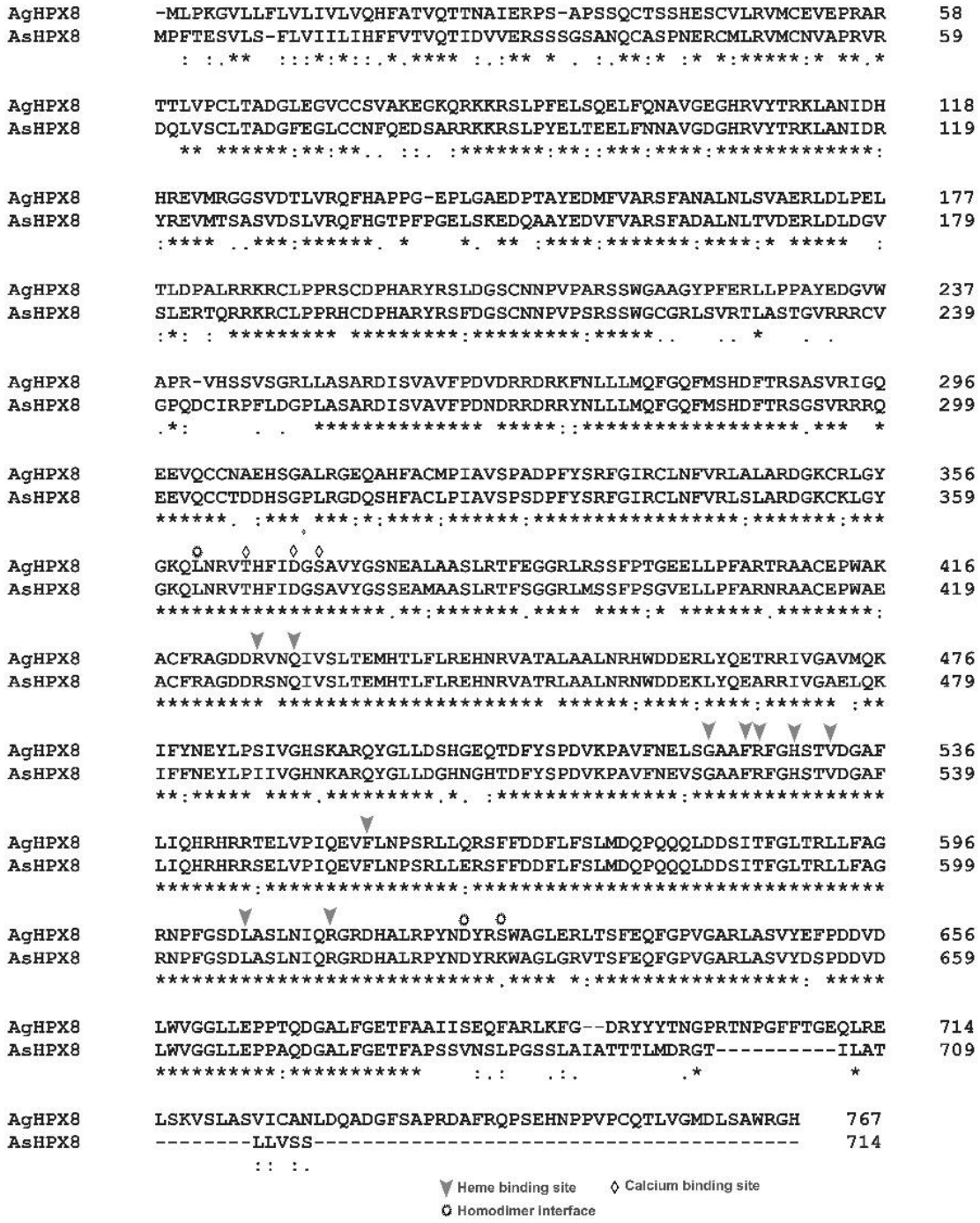


Fig 6.3: Conserve domains in AgHPX8 and AsHPX8 proteins. The conserved domain database (CDD) predicted numerous binding sites such as, heme binding, calcium binding and homodimer interface sites as represented by the characteristic symbols in the alignment between AgHPX8 and AsHPX8 proteins.

AgHPX8. These genes belong to animal heme-dependent peroxidases and bear all the characteristics - heme binding site, calcium binding site and homodimer interface necessary for their functioning.

6.3.4 Transcription factor binding sites in the regulatory region of AsHPX8 gene

To understand the regulation of AsHPX8 gene, we screened its regulatory region. The presence of promoter was analyzed in the randomly selected 1800 bp 5′ upstream region from the ATG of the predicted gene using the NNPP2.2 software. This program predicted the transcription start site (TSS) 33 bp upstream to the start codon, and it was assigned the position +1 (**Figure 6.4**). The presence of TATA box (TATAAA) was located at position -99 from the transcription start site. Further, we analyzed the putative transcription factor binding sites in its regulatory region using MatInspector and JASPAR software. The search criteria for these transcription factors binding motifs were defined within the insect family. Several transcription factors which regulate developmental processes, like homeobox, giant, etc. were identified (**Figure 6.4**). Importantly, grainyhead, Ecdysone, TGIF (TG-interacting factor), OVO group and chorion factor transcription factor binding sites were also predicted by the software. The TGIF transcription factor in *Drosophila* consists of two tandemly-repeated genes, achintya (Dmachi) and vismay (Dmvis), that act as transcriptional activators in the *Drosophila* spermatogenesis (Ayyar et al., 2003). In the *Bombyx mori*, TGIF is predominantly expressed in the testes and ovaries (Zhang et al., 2012). The grainyhead transcription factor is implicated in epithelial formation, as well as wound repair pathway in *Drosophila* and hence is required in developmental process (Mace et al., 2005). The OVO transcription factor encodes for a zinc finger protein that is required for female germline development in *Drosophila* (Mevel-Ninio et al., 1991) (Oliver et al., 1987) (Hayashi et al., 2017) and the ecdysteroid ecdysone is the molting hormone (Raikhel et al., 2002; Gilbert, 2004). In larvae, ecdysone is synthesized by the prothoracic glands, and in case of blood-fed female mosquitoes, it is induced in epithelial cells of the ovarian follicle (Hagedorn, 1989; Raikhel et al., 1999). It is noteworthy to mention here that *Anopheles* eggs are laid on the water surface and are sensitive to desiccation (Clements, 1992). Hence, they are covered with a protective chorion or eggshell- a chitinous covering and its rapid maturation (through a. tanning process associated with

melanin deposition: phenoloxidase-mediated chorion melanization and b. peroxidase-catalyzed chorion protein crosslinking) take place shortly after oviposition (Li, 1994).

In addition, the SignalP analysis of AsHPX8 protein revealed the presence of a signal

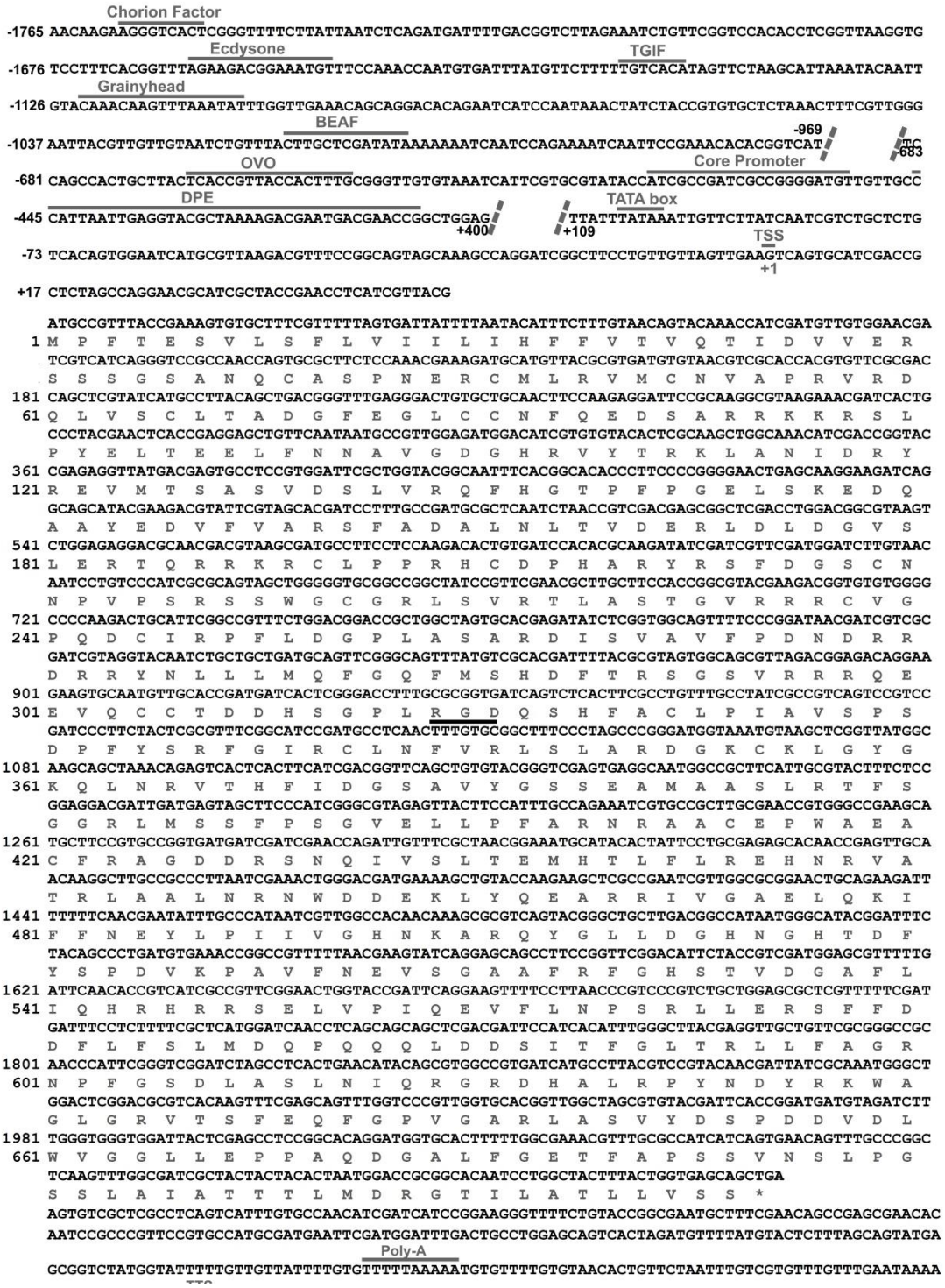


Figure 6.4: Analysis of regulatory elements in AsHPX8 gene. The cDNA (2313 bp) encodes a protein of 770 amino acid residues (residue number mentioned on the left). The figure depicts the amplified CDS and predicted UTRs. Transcription start site (TSS) is denoted by +1. Polyadenylation (Poly-A) and transcription termination site (TTS) are indicated with an underline. Binding sites for various transcription factors such as Ecdysone, Grainyhead, Chorion factor, OVO and TGIF are depicted in the regulatory region of AsHPX8 gene as predicted by MatInspector and JASPAR software. The integrin binding motif RGD is underlined.

peptide (1-24 amino acids) at the N-terminal (**Figure 6.4**). The cleavage site for signal peptidase (SPase) is present between the amino acids 24 and 25 (VQT_{24}-$I_{25}D$, the D=0.833. D cutoff=0.450) indicating that AsHPX8 is a secreted protein.

6.3.5 AsHPX8 is an Arthropod-specific gene

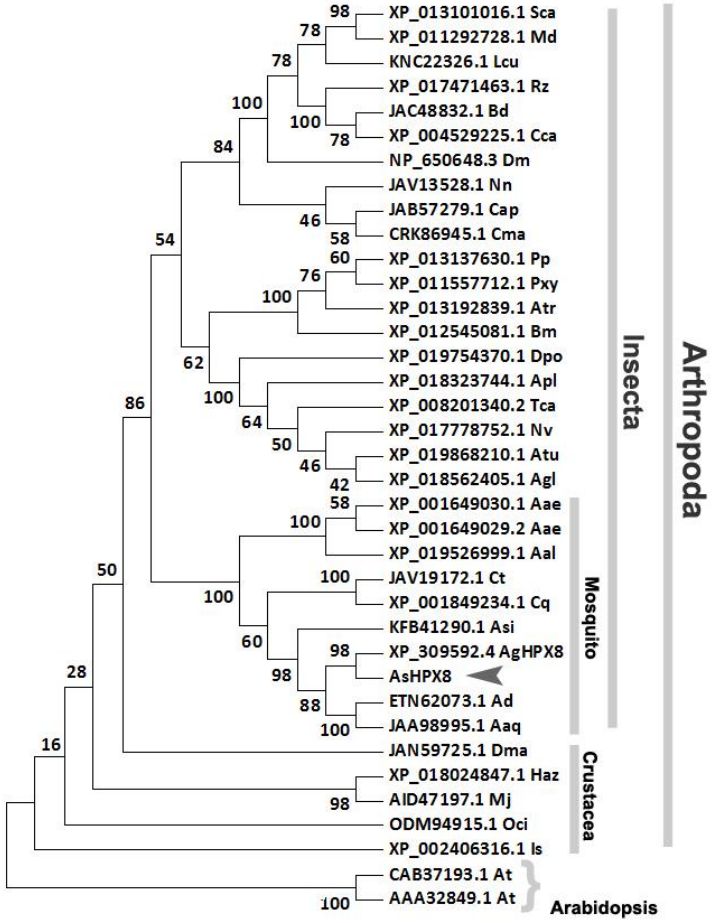


Figure 6.5: Phylogenetic tree analysis of AsHPX8 protein. The Maximum-Likelihood (ML) method in MEGA 7.0 was used to construct the phylogenetic tree of AsHPX8 with other heme peroxidases (HPX) proteins selected from different organisms as mentioned in **Table 2.4**. Arrowhead indicates AsHPX8 protein. The scale bar represents base substitutions per site. The numbers on the branches represent the % of 1000 bootstraps.

The AsHPX8 amino acid sequence was BLAST searched to NCBI database, and we selected thirty-three matching sequences to construct the phylogenetic tree (Table 2.4). The tree was generated by Maximum Likelihood method with 1000 bootstrap replicates in MEGA7.0. We observed that AsHPX8 is uniquely found only in the organisms belonging to Phylum Arthropoda (Figure 6.5).

Insect eggs are characterized by hardening of eggshell or chorion layer that protects against adverse environmental conditions and pathogens. The cross-linking of chorion protein is accomplished by peroxidases with the DUOX enzyme supplying the H_2O_2 (Dias et al., 2013). Also, AgHPX8 was reported to be involved in mosquito oogenesis (Emami et al., 2017; Marinotti et al., 2006). Hence, the presence of HPX8 in Phylum

Arthropoda can be attributed to its possible role in development, male spermatogenesis, female germ line development and egg chorion hardening.

6.3.6 AsHPX8 orthologs are present in worldwide distributed anophelines

The putative AsHPX8 ortholog contigs (or Ensembl identifiers) were retrieved from the genome of 17 other anophelines using nucleotide sequence of full-length AsHPX8 as a query. HPX8 contigs and total amino acids in HPX8 proteins for the worldwide distributed *Anopheles* are mentioned in **Table 6.1**. The percentage amino acid identities between the HPX8 proteins are mentioned in **Table 6.2**. Results shown in Figure 6.6 showed that HPX8 protein is present in all the anophelines distributed worldwide.

Table 6.1: List of putative AsHPX8 peroxidases retrieved from different species of *Anopheles* mosquitoes. The putative HPX8 peroxidase contig (or GenBank accession no.) from the genome of different anophelines were retrieved using nucleotide sequence of AsHPX8 (GenBank: KY363391) as a query. The overhanging sequences of the contigs were trimmed based on AsHPX8 sequence as mentioned in the Materials and Methods.

Vectorial capacity	*Anopheles* species (abbreviation)	Gene ID/ GenBank acc. No./Contig		Nucleotides in HPX8	Amino acids in HPX8 protein
Major vectors	*An. stephensi* (As)	KY363391		2313	770
	An. gambiae (Ag)	AGAP004038		2515	767
	An. arabiensis (Aar)	APCN01002644		2298	765
	An. atroparvus (Aat)	AXCP01001792		2301	721
	An. culicifacies (Acu)	AXCM01009432		2316	771
	An. darlingi (Ada)	ADMH02001557		2376	791
	An. dirus (Adi)	APCL01002393		2542	769
	An. farauti (Afa)	JXXC01026429		2506	767
	An. funestus (Afu)	APCI01005126		2415	804
	An. maculatus (Ama)	LULA01004755		2160	719
	An. sinensis (Asi)	ATLV01016358		2301	766
Minor vectors	*An. albimanus* (Aal)	APCK01001528		1722	573
	An. epiroticus (Aepi)	APCJ01000036		2298	765
	An. melas (Amel)	AXCO02017699		2271	756
	An. merus (Amer)	AXCQ02000904		2576	767
	An. minimus (Amin)	APHL01000075		2542	768
Non-vectors	*An. quadriannulatus* (Aqua)	APCH01002602		2552	767
	An. christyi (Achr)	APCM01001029	2295	764	

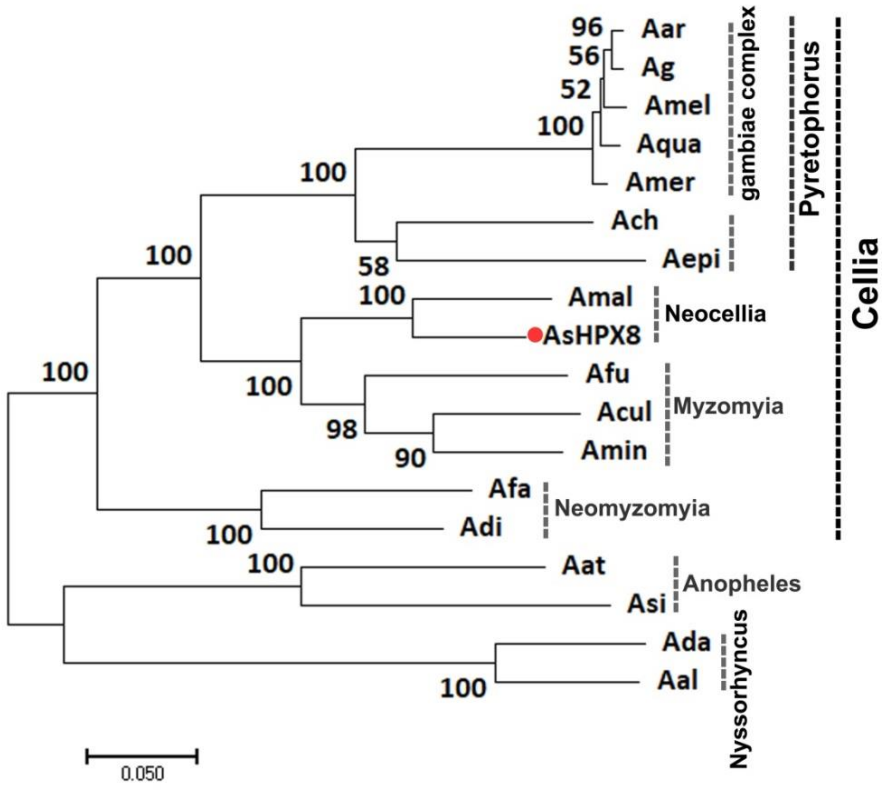


Figure 6.6: Phylogenetic analysis of HPX8 across worldwide distributed anophelines. The Maximum Likelihood (ML) method was used to construct the phylogenetic tree of AsHPX8 with other HPX8 from worldwide distributed anophelines (**Table 6.1**). The red circle indicates AsHPX8. The scale bar represents base substitutions per site. The numbers on the branches represent the % of 1000 bootstraps.

6.3.7 AsHPX8 is a secreted globular protein

Our analysis revealed that AsHPX8 gene contains an open reading frame (ORF) of 2313 bp that encodes for a protein 770 amino acids long. We also predicted the secondary structure of AsHPX8 protein using the Phyre[2] server. The resultant output revealed that AsHPX8 contains 44% alpha helix and 1% beta sheets (**Figure 6.7**). This indicated that AsHPX8 is a globular protein, as this category of proteins contains more alpha helices than beta sheets (Pace and Scholtz, 1998; Kajla et al., 2016a).

Since, the three-dimensional structure can be a valuable indicator of protein structure and function (Edwards and Cottage, 2003), we built the three-dimensional structure using the Phyre[2] server which selected human myeloperoxidase (PDB ID 3FAQ) as a template, with 100% confidence and 32% identity (**Figure 6.8A**). The active site consists of ARG_{273} and HIS_{492} as predicted by 3DLigandSite software using NAG (N-acetyl D-glucosamine) as heterogen (**Figure 6.8B)**.

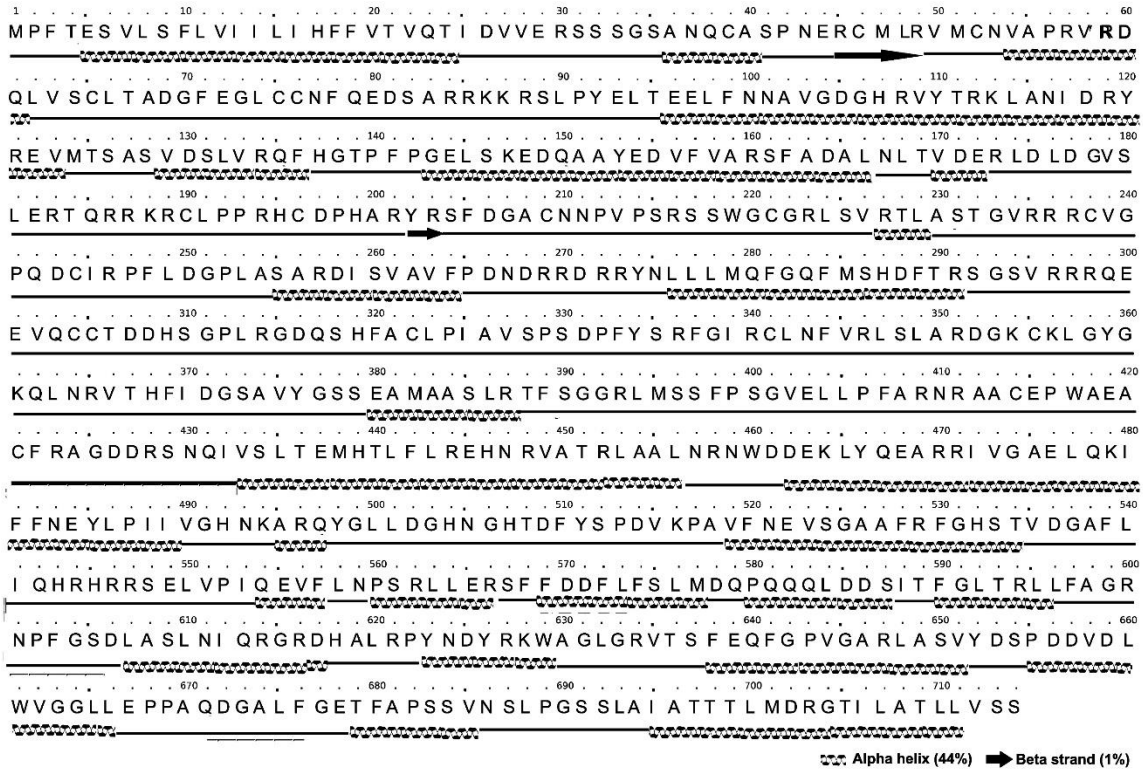


Figure 6.7: Deduced secondary structure of AsHPX8 protein. AsHPX8 protein contains 32% disordered, 43% alpha helix (represented by spirals) and 1% beta sheets (shown by arrows) as predicted by Phyre[2] software.

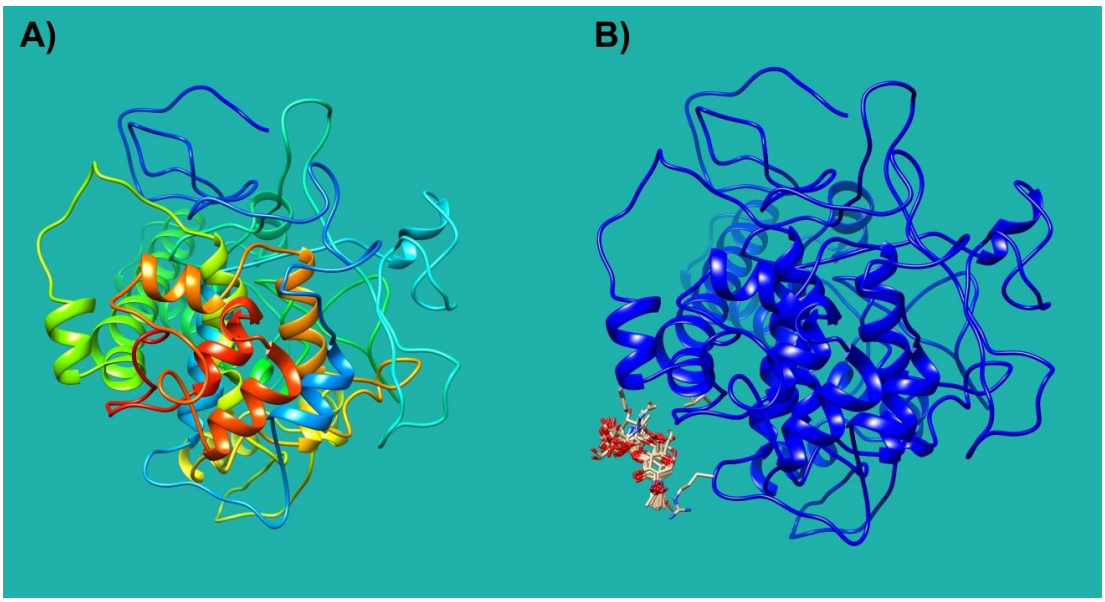


Figure 6.8: 3D structure analysis of AsHPX8 protein. A. AsHPX8 protein 3D structure was predicted by Phyre[2] software and B. ligand binding site was predicted by 3DLigand site server.

6.3.8 Expression analysis of AsHPX8 gene

6.3.8.1 AsHPX8 gene is induced in various stages of mosquito development

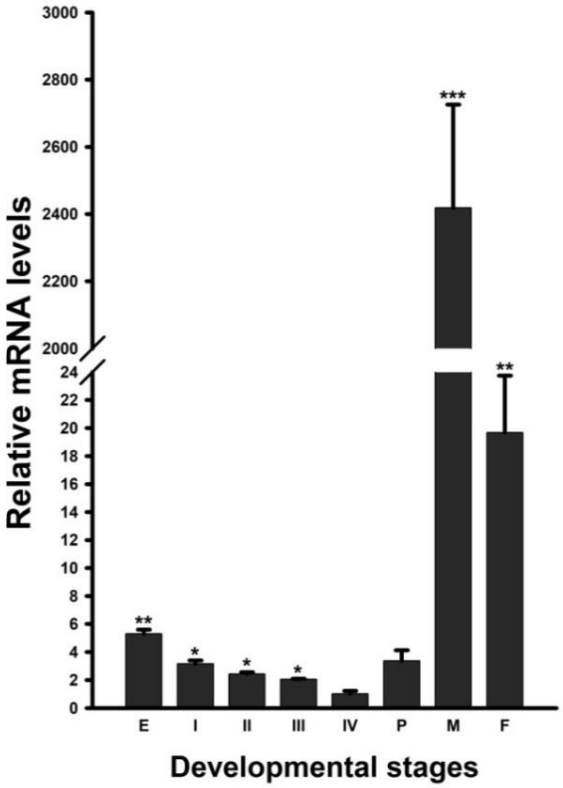


Figure 6.9: Relative AsHPX8 mRNA levels in different developmental stages. Results are representing the mean ± SD of relative mRNA levels in different developmental stages of *An. stephensi*. The mRNA expression levels of 4th larval instars (IV) were considered as controls. Significant differences ($p \leq 0.05$, $p \leq 0.001$ and $p \leq 0.0001$) in the relative mRNA levels of different stages against control are indicated by one, two or three asterisks (*) respectively.

Heme peroxidases are previously reported both in adult male and female mosquitoes with several roles in physiology, fecundity, survival or immune response (Shaw et al., 2014; Kumar et al., 2010a; Kajla et al., 2017). Hence, we analyzed the developmental stage-specific expression pattern of AsHPX8 gene in all the stages of mosquito development, i.e., eggs, all four stages of larvae (I, II, III, IV), pupae and adult males and females.

Semi-quantitative real-time PCR (qPCR) analysis indicated that AsHPX8 gene was expressed in all these stages of development (**Figure 6.9**). The mRNA expression levels were ~6-fold (p=0.0069) in eggs, ~3-fold in Ist (p=0.0203), ~2-fold in IInd (p=0.0275) and IIIrd (p= 0.0397) instar larvae, ~3-fold in pupae against 4th larval instars taken as 1 (IV). Interestingly, the expression levels of AsHPX8 gene increased to ~2400-fold in males (p≤0.0001) and ~20-fold in females (p≤0.001) against 4th larval instars (IV). These results indicated that AsHPX8 gene might play a crucial role in adult mosquitoes. Also, the AsHPX8 regulatory region had binding sites for OVO, TGIF transcription factors. This revealed its possible role in male spermatogenesis and females germ line changes. These also explain the relatively high mRNA levels of AsHPX8 gene in adult males and females.

6.3.8.2 Differential expression of AsHPX8 gene in the different mosquito body compartments

The relative mRNA levels of AsHPX8 gene were analyzed in sugar-fed, and 24 h post blood fed midguts or carcasses to elucidate its mosquito body compartment-specific expression. The result presented in **Figure 6.10** showed that AsHPX8 expression levels were ~3-fold ($p \leq 0.001$) higher in blood-fed midguts than sugar-fed midguts. Also, its expression increased by ~7.5-fold ($p \leq 0.0001$) in 24 h post blood fed carcasses as compared to sugar fed carcasses. Hence, AsHPX8 gene had a higher expression in 24 h blood-fed midguts and carcasses as compared to their respective sugar fed midguts and carcasses.

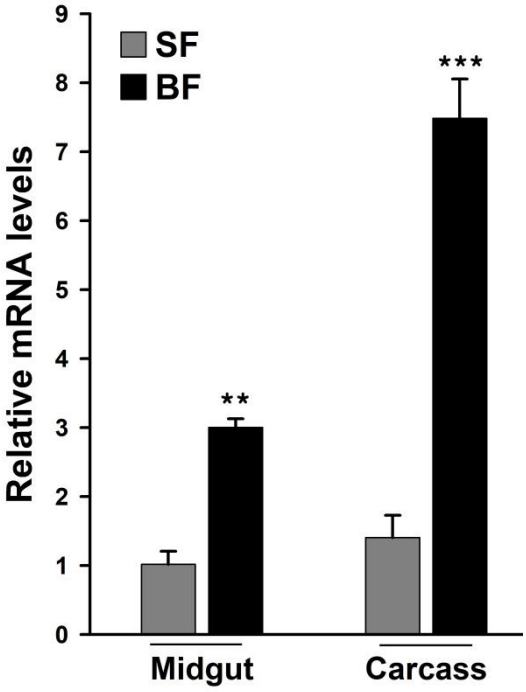


Figure 6.10: Relative expression of AsHPX8 gene in different mosquito body compartments. Relative mRNA levels of AsHPX8 gene were analyzed in midguts and carcasses of sugar fed or 24 h post blood fed mosquitoes. Significant differences $p \leq 0.0001$ or $p \leq 0.001$ are indicated by three or two asterisks (*),

These results can be interpreted as follows. The CDD analysis of AsHPX8 protein revealed its peroxinectin like nature- RGD motif for integrin binding (**Figure 6.4**). Peroxinectins are involved in the invertebrate immunity and are homologs of vertebrate myeloperoxidase. They are involved in cell adhesion mediated by their integrin binding motif(s) and antibacterial responses by the enzymatic reaction with H_2O_2 and halide that lead to the formation of hypochlorous acid (toxic to the pathogen) (Klebanoff, 1968; Allen and Stephens, 2011). The relatively higher levels of AsHPX8 gene in blood fed midguts as compared to sugar fed midguts showed that the gene was induced post 24 h blood feeding (**Figure 6.10**), as reported for another *An. stephensi* HPX - AsHPX15 which is also peroxinectin in nature (Kajla et al., 2016b).

Also, transcription factor binding analysis by MatInspector predicted AsHPX8 gene upstream regulatory region to have a binding site for OVO transcription factor. OVO is reported to be involved in female germline development, and thus AsHPX8

gene may be induced in the blood fed carcasses as the process of blood feeding initiates ovarian follicle development via accumulation of vitellogenin yolk proteins and in our studies, the carcass tissue refers to all mosquito tissues except midgut (Hayashi et al., 2017).

6.3.8.3 Expression kinetics of AsHPX8 gene in sugar-fed mosquito midguts

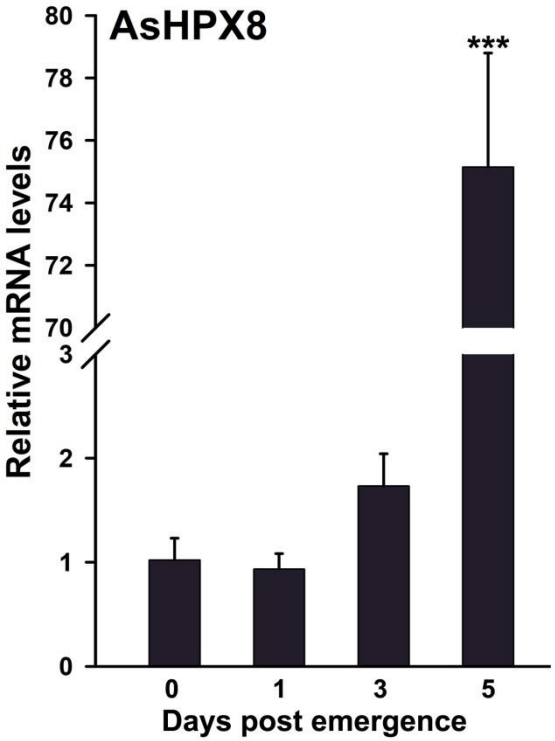


Figure 6.11: Relative mRNA levels of AsHPX8 gene in sugar-fed midguts. The relative expression of AsHPX8 gene was analyzed in adult female midguts at different days post emergence. Newly hatched sugar fed midguts at 0 day (d) was taken as 1. Significant difference $p \leq 0.0001$ is indicated by three asterisks (*).

6.3.8.4 Based on our previous results, we wanted to explore the regulation of this gene in newly emerged adult female mosquito midguts. The results presented in **Figure 6.11** shows that there was a significant induction of ~75-fold ($p<0.0001$) in AsHPX8 mRNA expression levels 5 days (d) post-emergence against 0 days i.e. immediately post emergence from pupal shell.

Further, we analyzed the expression of 16S rRNA levels in these samples to investigate the induction of AsHPX8 gene. Results presented in **Figure 6.12** shows that 16S rRNA gene was induced by ~13-fold ($p<0.0001$) in adult female midguts 5d post-emergence against 0-day mosquito midguts.

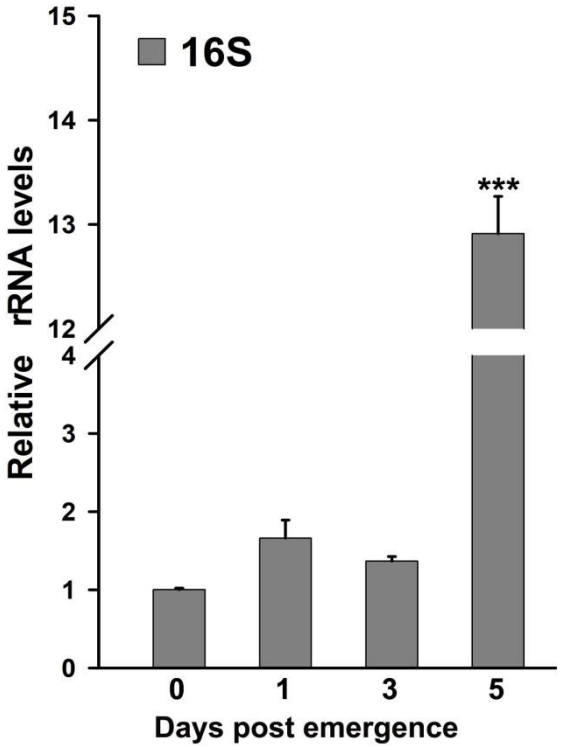


Figure 6.12: Relative levels of 16S rRNA in sugar fed adult mosquitoes post emergence from pupa. The relative expression of 16S rRNA was analyzed in adult midgut tissues collected at different time points post emergence. The sugar fed midguts at 0d were taken as controls. Significant differences $p \leq 0.001$ is indicated by three asterisks

It is presumed that midgut bacteria are acquired through vertical inheritance as well as from the surrounding environments (Minard et al., 2013; Buck et al., 2016). Hence, the up-regulation of 16S rRNA in the midguts of adult females 5d post-emergence directly implied an increase in bacterial population with time. The induction of AsHPX8 gene at the same time point indicated that AsHPX8 gene was induced simultaneously with an increase in gut bacteria.

6.3.8.5 Expression of AsHPX8 in the blood-fed midgut tissues of adult female mosquitoes

Further, we analyzed the relative levels of 16S rRNA, and AsHPX8 gene post blood fed midgut time kinetics samples. We found that the expression of 16S rRNA increased to ~1800 fold (p=0.0007) at 6 h which decreased to ~150-fold (p=0.0004) at 12 h and increased to ~250-fold (p=0.0005) at 18 h and ~9000 (p=0.0002) fold post 24 h of blood feeding, against sugar-fed midguts as control (**Figure 6.13**).

This up-regulation of 16S rRNA gene correlated with the probable proliferation of endosymbiotic midgut bacteria post blood feeding. A similar pattern of induction was observed in the mRNA levels of AsHPX8 gene at the same time points viz. 6 h (p=0.0004) and 24 h (p=0.0002) against sugar-fed midguts as a control. This implied that AsHPX8 gene was up-regulated concomitantly with bacteria post blood feeding in *An. stephensi* midguts.

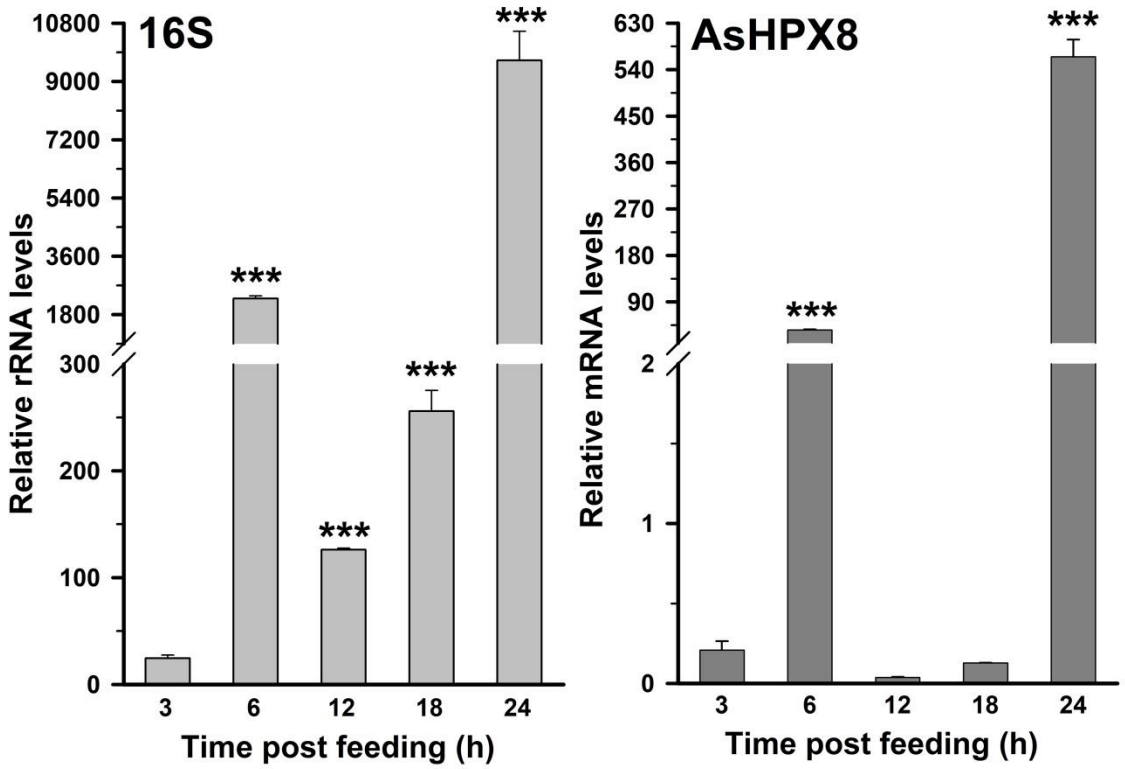


Figure 6.13: Relative expression of 16S rRNA and AsHPX8 gene in blood-fed midguts of *An. stephensi* female mosquitoes. Relative rRNA and mRNA expression levels of 16S and AsHPX8 respectively, in blood-fed midguts of adult female mosquitoes. Significant differences $p < 0.0001$ are indicated by three asterisks (*).

6.3.8.6 AsHPX8 gene is induced in blood fed mosquito carcasses

To explore the role of AsHPX8 in oogenesis, we blood fed naïve sugar fed uninfected mosquitoes and collected carcasses at various time points. DUOX is reported to generate ROS (Ha et al., 2009) which are then utilized by other heme peroxidases. Thus, we evaluated the relative mRNA levels of AsHPX8 and AsDUOX genes in these tissue samples. We found that AsHPX8 gene was induced by 6-fold, ~20-fold, ~35-fold at 24 h, 48 h and 72 h respectively, and then reduced to ~8-fold at 96 h post blood feeding against sugar fed mosquito carcasses (**Figure 6.14**). A similar pattern of expression was observed for AsDUOX gene {which is reported to be involved in mosquito egg development by Dias et al. (2013)} with induction of ~2-fold (p=0.0021), ~7-fold (p=0.0002) and ~17-fold ($p \leq 0.0001$) at 24 h, 48 h and 72 h, respectively against sugar fed carcasses (**Figure 6.14)** (Dias et al., 2013). This indicated that AsHPX8 and AsDUOX genes are possibly involved in *An. stephensi* egg development and maturation.

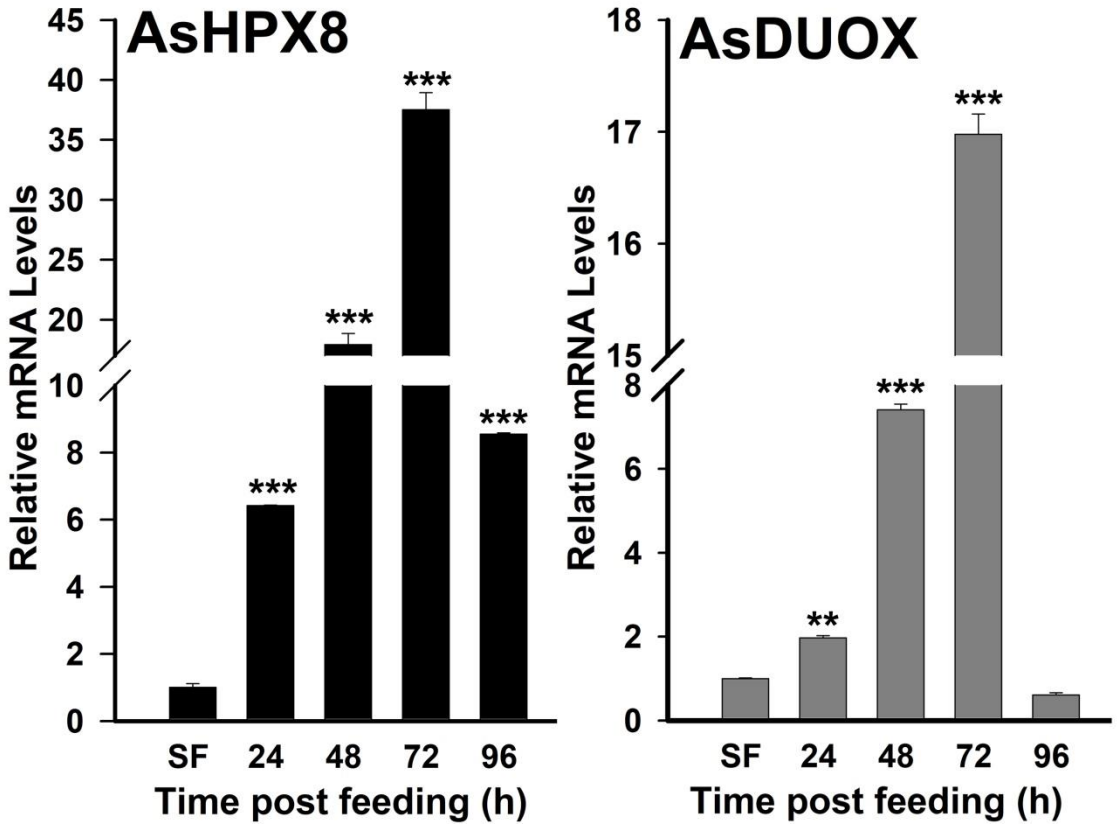


Figure 6.14: Relative expression of AsHPX8 and AsDUOX genes in blood-fed carcasses of *An. stephensi* mosquitoes. Relative mRNA levels of AsHPX8 and AsDUOX genes were analyzed in *An. stephensi* carcasses at different time points after blood feeding. Relative fold induction was calculated against sugar fed (SF) carcasses. Significant differences $p \leq 0.0001$, $p \leq 0.001$ are indicated by three or two asterisks (*), respectively

6.3.8.7 Expression analysis of AsHPX8 in bacteria fed midguts

6.3.6.6.1 Exogenous bacteria fed with saline

As we observed in **Figure 6.11**, the relative levels of AsHPX8 gene were higher in the sugar-fed midguts 5d post-emergence, as well as it was induced post blood feeding (**Figure 6.13**), we were interested to analyze AsHPX8 gene expression upon bacteria feeding. In this context, we allowed mosquitoes to artificially feed either on saline alone (control) or saline supplemented with a mixture of exogenous bacteria [(Gram-negative *E. coli* and Gram-positive *M. luteus* (total $1x10^9$ cells/ml)].

To confirm successful bacterial infection, we analyzed the expression of 16S rRNA in the bacteria fed midguts. Results presented in **Figure 6.15** show that bacterial population increased by 11000-fold at 2 h, 30000-fold at 6 h, 1200-fold at 12 h and 69000-fold at 18 h against their saline fed midguts (controls), respectively.

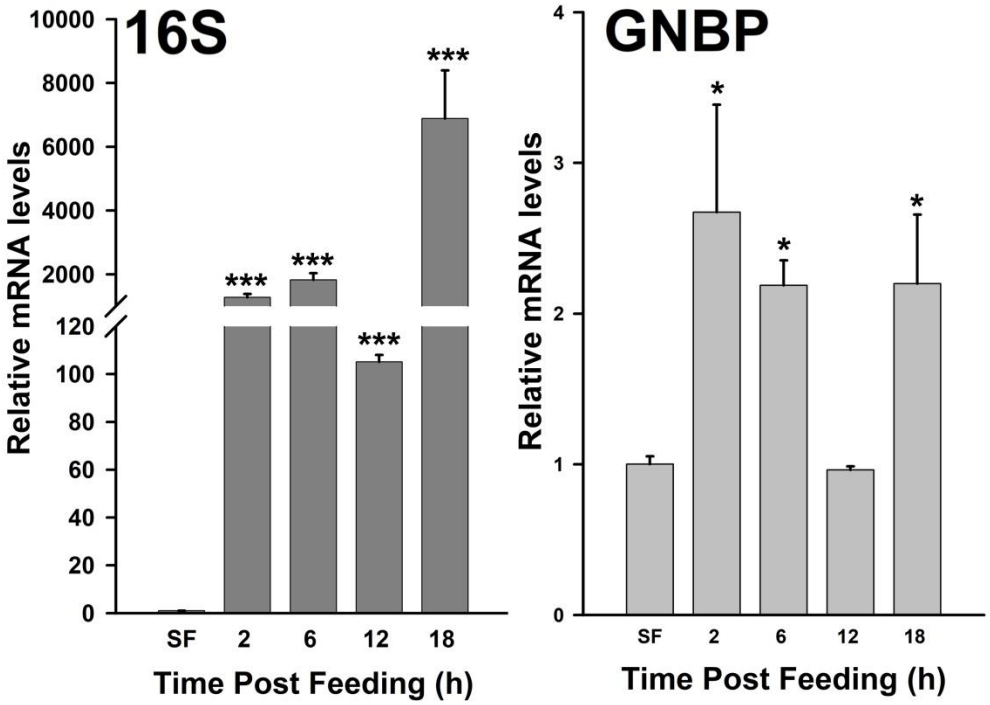


Figure 6.15: Relative expression of 16S rRNA and GNBP genes in saline supplemented with exogenous bacteria fed adults midguts. The expression of 16S rRNA and GNBP genes were analyzed in their exogenous bacteria fed with saline fed midguts at various time points. SF represents the sugar fed midguts and taken as 1. Relative levels are presented against respective control at each time points. Significant difference $p \leq 0.0001$ $p \leq 0.05$ in relative rRNA levels of 16S and mRNA levels of GNBP are indicated by three or one asterisks (*).

To determine the activation of mosquito immunity against the bacteria, we analyzed the expression of a known PRR (Pattern Recognition Receptor) - GNBP (Gram-Negative Bacteria Binding Protein) gene. GNBP gene was previously reported to be transcriptionally up-regulated in the presence of bacterial elicitor like lipopolysaccharide

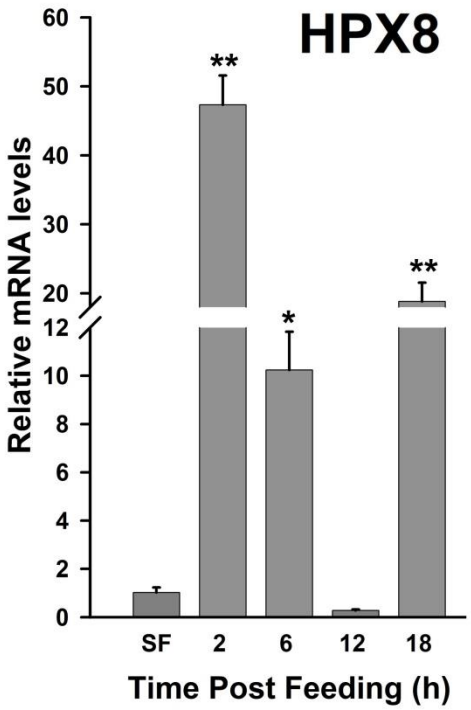


Figure 6.16: Relative expression of AsHPX8 gene in saline supplemented with exogenous bacteria fed adults midguts. The expression of AsHPX8 gene was analyzed in their exogenous bacteria fed with saline fed midguts at various time points. SF represents the sugar fed midguts and taken as 1. Relative levels are presented against respective control at each time points. Significant difference $p \leq 0.001$ or $p \leq 0.05$ in relative mRNA levels of AsHPX8 are indicated by two or one asterisks

(Dimopoulos et al., 1997; Clayton et al., 2014). Results presented in **Figure 6.15** revealed that GNBP gene was significantly up-regulated in bacteria supplemented saline fed midguts by ~3-fold at 2 h (p=0.0057), ~2-fold at 6 h (p=0.0131), and ~2-fold at 18 h against saline fed midguts.

Further, we analyzed the regulation of AsHPX8 gene in the saline supplemented with exogenous bacteria fed midguts. We observed that the gene was induced by ~47-fold at 2 h (*p*=0.0034), ~10-fold at 6 h (*p*=0.0124), and reduced drastically at 12 h below basal expression levels (*p*=0.0356), and ~19-fold at 18 h (*p*=0.0074) in saline supplemented with bacteria fed midguts against control midguts (**Figure 6.16**). These results indicated that AsHPX8 gene was induced due to heavy load of exogenous bacteria in the midgut.

6.3.6.6.2 Exogenous bacteria fed with blood

6.3.6.6.3 Further, we investigated the regulation of AsHPX8 gene in the presence of exogenous bacteria fed with blood, as blood feeding itself induces proliferation of endosymbiotic midgut bacteria and AsHPX8 gene was induced post blood feeding (**Figure 6.13**). Here, adult female mosquitoes were allowed to feed on either blood alone (control) or blood supplemented with bacteria (test) and midguts were collected at different time points as

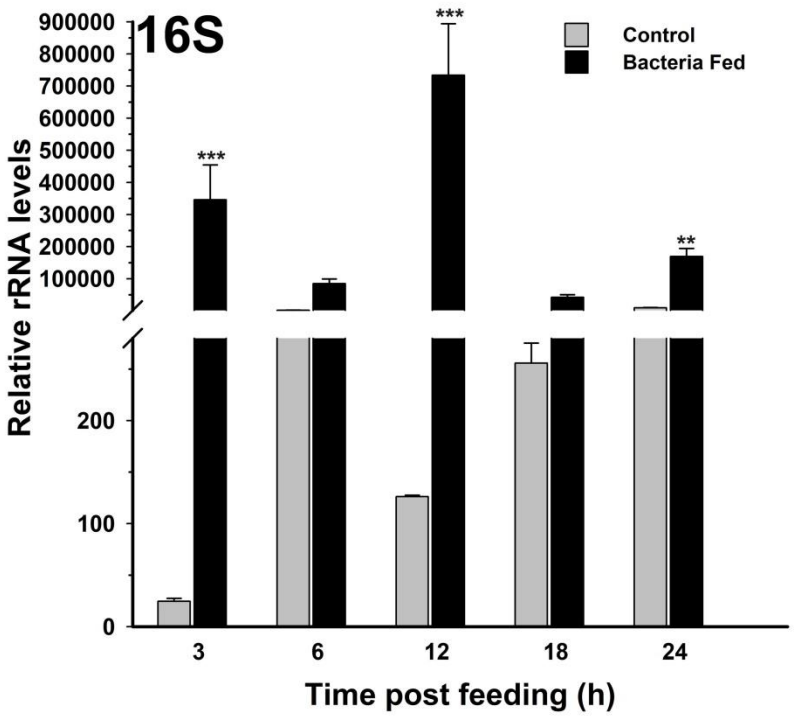
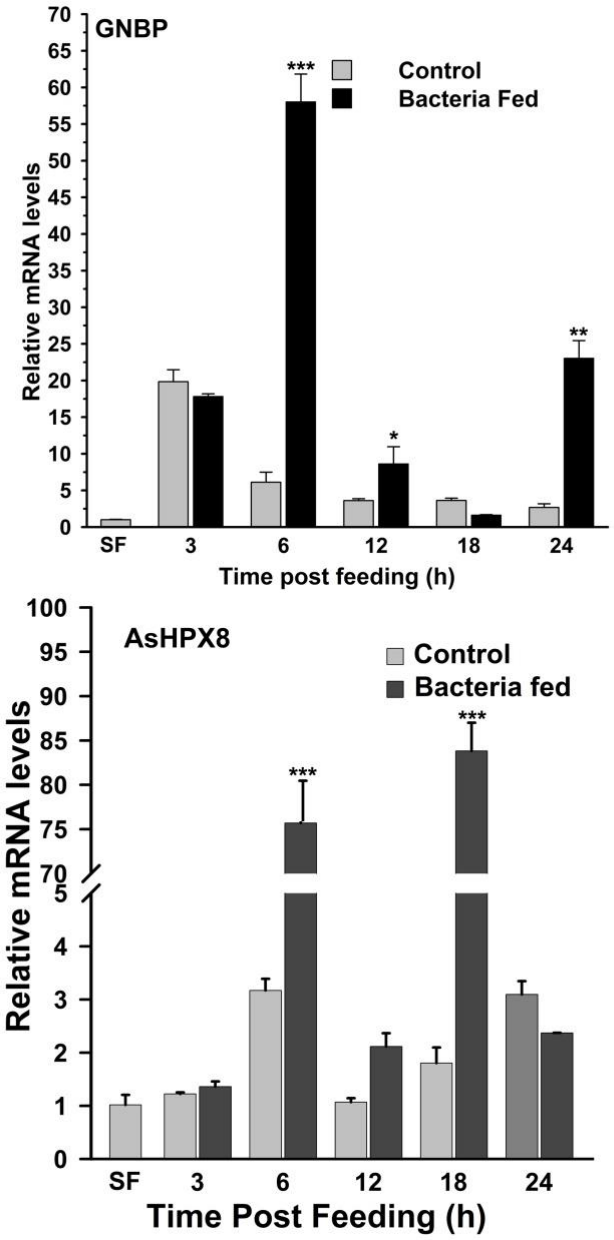


Figure 6.17: Relative rRNA levels of 16S in blood fed and blood supplemented with bacteria fed adult mosquito midguts at various time points. The expression kinetics of 16S rRNA was analyzed in their blood supplemented with bacteria-fed midguts against sugar-fed midguts. Relative levels are presented against respective control at each

described in Material and Methods. The bacterial growth was analyzed in controls, and exogenous bacteria fed midguts through 16S rRNA analysis.

The 16S rRNA levels presented in **Figure 6.17** revealed growth of exogenous bacteria in the bacteria supplemented blood fed midguts against their respective blood fed midguts. These results are in agreement with previous findings where an increase in endogenous bacteria was observed after 6 h of blood feeding and that further followed the similar growth pattern (Kumar et al., 2010b; Luckhart et al., 1998). In case of bacteria supplemented blood-fed midguts, altered levels of 16S rRNA at different time points indicated that the growth of bacteria is regulated rhythmically, most probably by the midgut immunity. In these samples expression of GNBP (antibacterial gene) was evaluated to analyze the successful activation of mosquito antibacterial immunity.



The results shown in **Figure 6.18** revealed that GNBP gene was induced by ~10-fold at 6 h, 2-fold at 12 h, and 9-fold at 24 h in bacteria supplemented blood fed midguts in comparison to the respective blood fed controls.

Also, we analyzed the regulation of AsHPX8 gene in the blood supplemented bacteria fed midguts. We

Figure 6.18: Relative mRNA levels of GNBP and AsHPX8 genes in blood fed and blood supplemented with bacteria fed adult mosquito midguts at various time points. The relative expression of GNBP and AsHPX8 genes were analyzed in the blood supplemented with bacteria-fed midguts against sugar-fed midguts. Relative levels are presented against respective control at each time points. Significant difference $p \leq 0.0001$ or $p \leq 0.001$ or $p \leq 0.05$ in relative mRNA levels of GNBP and AsHPX8 genes are indicated

observed that the gene was significantly induced by 25-fold at 6 h and 40-fold at 18 h against blood fed midguts. However, its mRNA levels were unaffected by the bacterial feeding at 3 h, 12 h, and 24 h when compared to their respective blood fed controls. These data indicated that the mRNA levels of AsHPX8 are induced in the midgut in the presence of blood supplemented with exogenous bacteria or saline. This result can be explained with the reduced 16S rRNA expression at 6 h and 18 h (**Figure 6.19**).

6.3.9 HPX8 has a duplicated paralog named HPX7

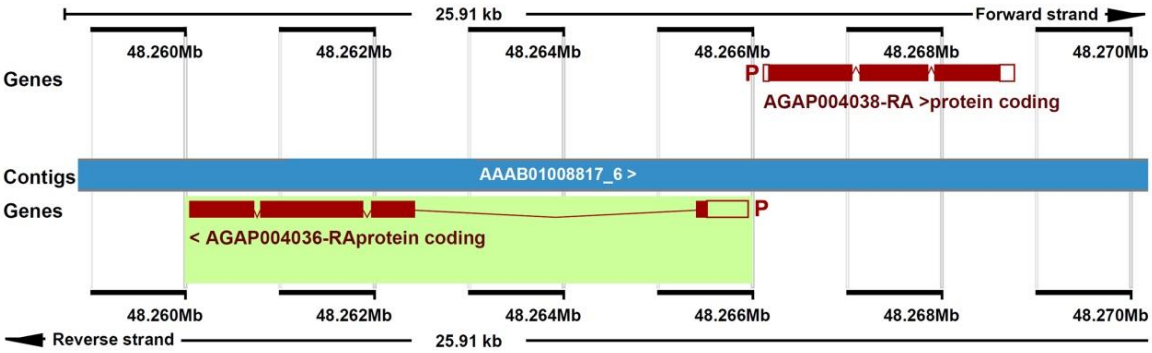


Figure 6.19: Head-to-head orientation of *An. gambiae* AgHPX8 and AgHPX7 genes sharing a possible bidirectional promoter. The figure shows that orientation of HPX8 and HPX7 in *An. aambiae*.

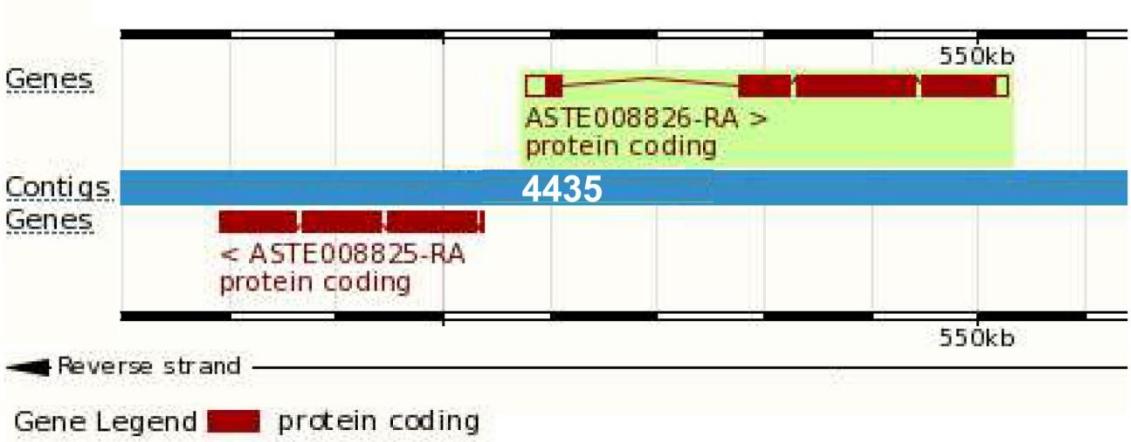


Figure 6.20: Head-to-head orientation of *An. stephensi* AsHPX8 and AsHPX7 genes sharing a possible bidirectional promoter. The figure shows that orientation of HPX8 and HPX7 in *An. stephensi*.

Initially, while attempting to predict promoter for AsHPX8, we found that in *An. gambiae* HPX8 (2R:48266124-48268778) and HPX7 (2R:48260053-48265961) are surprisingly close to each other in a head-to-head orientation, with an intergenic distance of 163 nucleotides (**Figure 6.19**). The Vectorbase reported AgHPX8 and AgHPX7 as paralogs

of each other with a 40.94% identity (**Figure 6.21A**). Hughes (2012) reported that HPX7 arose by gene duplication after the MRCA (Most Recent Common Ancestor) of Culicidae and Drosophilidae. HPX8 and HPX7 bifurcated from a single node during the evolution of heme peroxidases in various insects (Kajla et al., 2015b). This showed that

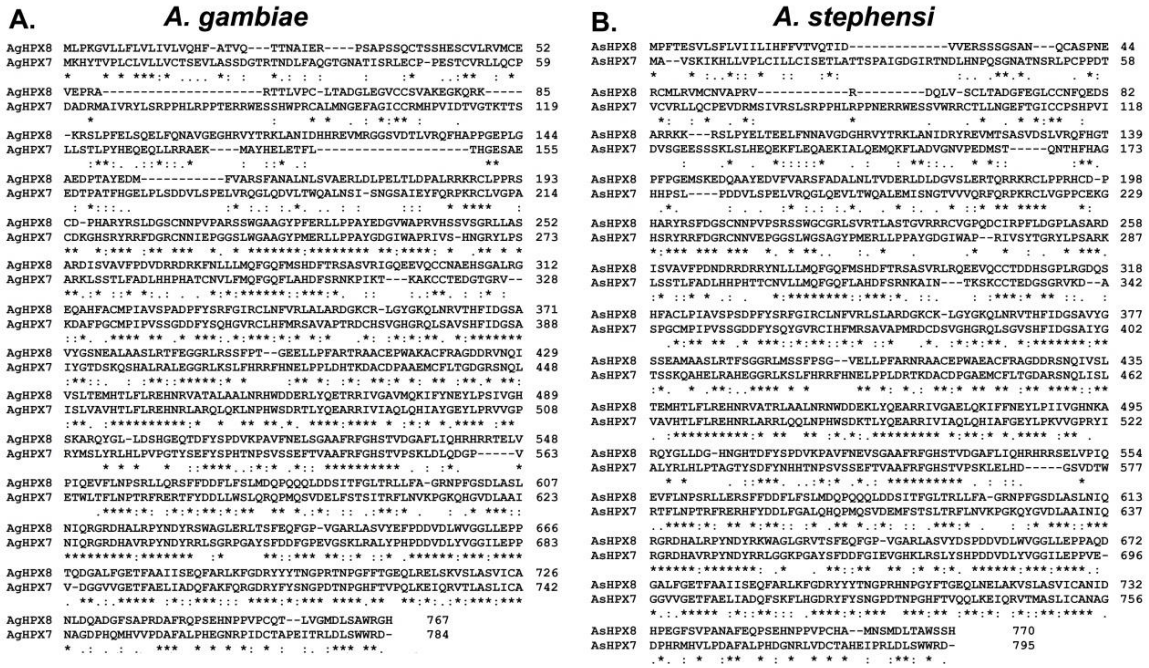


Figure 6.21: Amino acid alignment between A. AgHPX8 and AgHPX7 and B. AsHPX8 and AsHPX7 prepared using Clustal Omega.

they have originated from a single ancestral gene.

Similarly, we found that AsHPX8 and AsHPX7 are also very close to each other

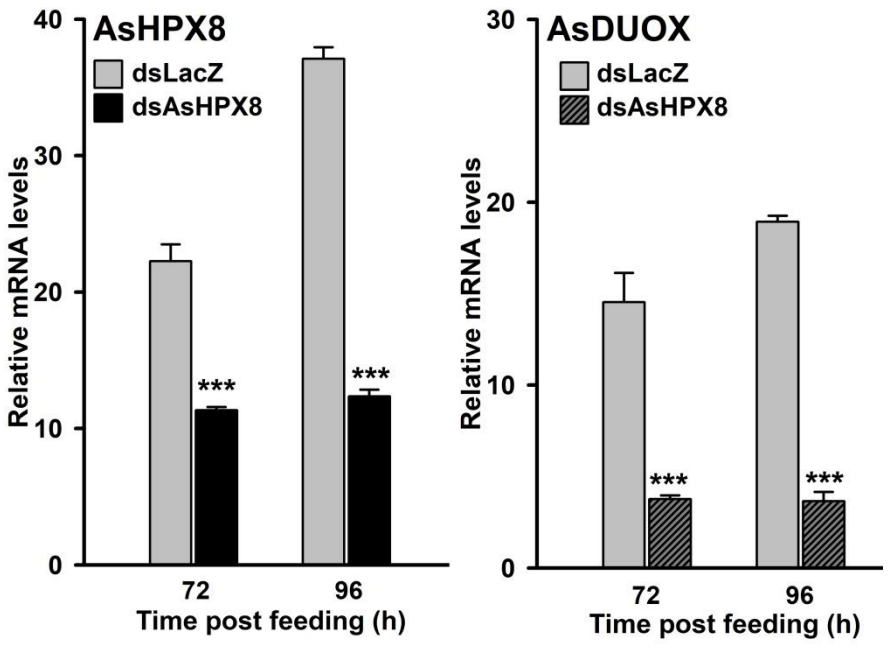


Figure 6.22: Relative mRNA levels of AsHPX8 and AsDUOX in AsHPX8 silenced blood-fed carcasses. The relative expression of AsHPX8 and AsDUOX genes were analyzed in the dsLacZ and dsAsHPX8 silenced blood-fed carcasses collected post 72 h and 96 h. Significant difference $p \leq 0.0001$ in relative mRNA levels of AsHPX8 and AsDUOX genes are indicated by three asterisks (*).

in a head-to-head orientation with an intergenic region of 407 bp (**Figure 6.20**). Moreover, they share an amino acid percentage identity of 40.64% and are reported as paralogs in Vectorbase (**Figure 6.21B**). Moreover,. Thus, AsHPX8 and AsHPX7 are duplicated paralogs.

6.3.8.8 AsHPX8 gene silencing activates its duplicated gene-AsHPX7

The mRNA levels of AsHPX8 gene were induced post 72 h and 96 h post blood feeding in the mosquito carcass as compared to sugar fed carcass (**Figure 6.14**). To decipher the role of AsHPX8 gene in mosquito oogenesis and/or chorion hardening, we carried the silencing of this gene. Briefly, we injected mosquitoes with LacZ (controls) or AsHPX8 (silenced) dsRNA, and blood fed them four days post dsRNA injection. The mosquito carcasses were collected post 72 h and 96 h of blood feeding. The percentage of gene silencing and effect on other downstream genes was analyzed as described in Material and Methods. The relative AsHPX8 mRNA levels in dsLacZ injected controls and AsHPX8 silenced

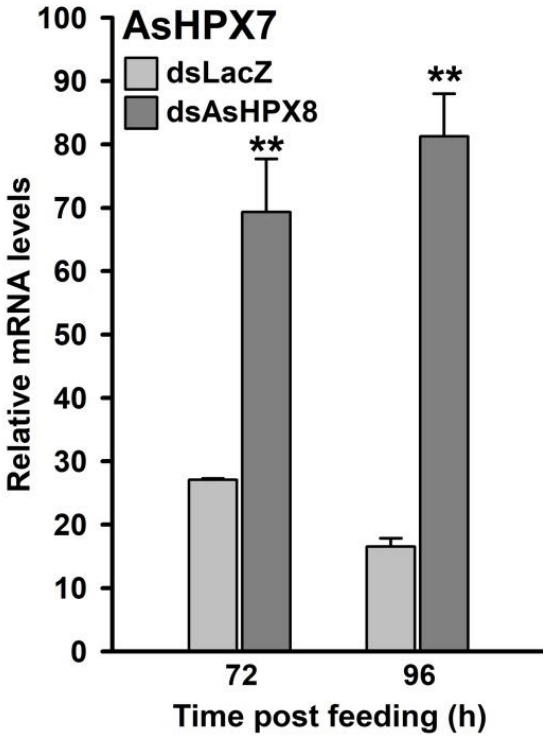


Figure 6.23: Relative mRNA levels of AsHPX7 gene in AsHPX8 silenced 72 h and 96 h post blood fed adult mosquito carcasses. The relative expression of AsHPX7 gene was analyzed in the dsLacZ and dsAsHPX8 silenced post 72 h and 96 h blood-fed carcasses. Significant difference $p \leq 0.001$ in relative mRNA levels of AsHPX7 gene is indicated by two asterisks (*)

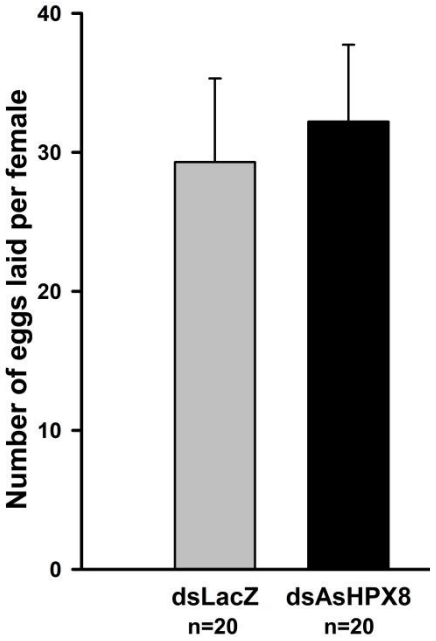


Figure 6.24: Oviposition pattern of AsHPX8 silenced blood fed mosquitoes. Mean percentage (± SE) of the total eggs laid by the dsLacZ and dsAsHPX8 injected blood fed mosquitoes. Mosquitoes were blood-fed on same mice and exposed to a 12 h day: light cycle.

carcasses revealed that we achieved 50% and 70% silencing of this gene at 72 h and 96 h, respectively (**Figure 6.22**). Also, we observed the down-regulation of AsDUOX gene upon AsHPX8 silencing.

Further, we analyzed the upstream regulatory region of AsHPX7 gene and found TGIF (-1901bp), OVO (-1237bp) and chorion transcription factor binding sites (-1454bp) (Ayyar et al., 2003) (Mevel-Ninio et al., 1991) (Oliver et al., 1987) (Hayashi et al., 2017). Hence, we analyzed the expression of AsHPX7 gene in these 72 h and 96 h post blood fed AsHPX8 silenced carcasses and we observed that AsHPX7 gene was induced by ~2.5 fold (p=0.0003) and ~4 fold (p=0.0004) at 72 h and 96 h post silencing against LacZ injected blood fed mosquito carcasses (**Figure 6.23**). Hence, this indicated that the AsHPX7 gene was induced in AsHPX8 gene silenced mosquito carcasses.

Moreover, we analyzed the effect of silencing on the number of eggs laid by *An. stephensi* females, and we observed no significant differences in egg laying responses (**Figure 6.24**). Thus, we hypothesize that AsHPX7 gene possibly counteracts the effects of AsHPX8 gene silencing.

6.4 Discussion

Heme peroxidases are crucial for insect growth, development, and immunity (Shi et al., 2012). In this chapter, we studied *An. stephensi* heme peroxidase HPX8. We found that it is a true ortholog of AgHPX8. Also, HPX8 has a duplicated counterpart in the genomes of *An. gambiae* and *An. stephensi* HPX7. HPX8 is found in Phylum Arthropoda, and this result correlates with it being a putative chorion peroxidase, as it has binding sites for chorion transcription factor (**Figure 6.4)**. Also, AsHPX8 is conserved among anophelines and share 65-99% amino acids identity (**Figure 6.8**, **Table 2.4 and 6.3**)

AsHPX8 gene is expressed in all the developmental stages of mosquito but showed peak induction in adult males and females (**Figure 6.11**) which indicated towards its important role in the adult development and reproduction. One of the key processes of insect development is ecdysis (i.e., molting), and this is triggered by the ecdysone hormone. We observed that AsHPX8 gene has ecdysone binding site in its upstream regulatory region. Thus it may be hypothesized that HPX8 is important in mosquito development (**Figure 6.9**). Our analysis also revealed the presence of N-

terminal signal peptide in AsHPX8 protein (**Figure 6.4)** which indicated that it is a secretory protein.

Moreover, AsHPX8 gene was induced 5-day postemergence in adult female midguts (**Figure 6.13**). Also, the gene was induced in *An. stephensi* midguts post 6 h and 24 h of blood feeding when there was an increase in midgut bacteria (**Figure 6.15**). The gene was also induced in bacteria supplemented with saline (**Figure 6.17**) or blood (**Figure 6.19**) fed mosquito midguts. Thus AsHPX8 plays a crucial role in midgut bacterial homeostasis. Also, we observed that AsHPX7 gene was induced upon AsHPX8 silenced blood fed carcasses. This explained the absence of any difference in egg laying between the control and AsHPX8 silenced carcasses.

6.5 Conclusion

This is the first report of identification and characterization of heme peroxidase HPX8 from *An. stephensi*. It shares conserved domains with its close ortholog in *An. gambiae* and is present only in Phylum Arthropoda. Its upstream regulatory analysis revealed various transcription factor binding sites that suggest the gene's possible role in mosquito development and reproductive cycle. Also, its induction in mosquito midguts and carcasses post blood feeding points strengthens our *in silico* analysis. Also, the silencing of this gene induced the expression of its duplicated paralog - AsHPX7.

Table 6.2: HPX8 amino acid percentage similarities among Anophelines – The percentage similarity were analyzed using Clustal Omega. The range of similarity is - 98.43% - 65.66%, shaded light and dark gray respectively. The column AA stands for HPX8 protein length in amino acids. The abbreviations for *Anopheles* species are same as mentioned in Table 6.1.

		AA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	**AgHPX8**	767		75.46	98.17	75.16	97.97	67.99	81.41	74.18	76.38	98.43	97.61	67.50	69.45	71.58	76.15	74.63	80.63	77.43
2	**AminHPX8**	758	75.46		75.59	87.92	75.25	68.64	77.56	75.39	75.57	75.09	75.35	69.03	69.19	71.51	81.70	84.82	76.64	82.24
3	**AquaHPX8**	787	98.17	75.59		75.10	97.82	67.91	81.53	74.06	75.99	98.00	97.90	67.51	69.30	71.24	76.05	74.98	80.66	77.52
4	**AculHPX8**	769	75.16	87.92	75.10		75.12	67.74	78.02	74.08	74.78	74.93	75.18	68.43	69.31	69.95	81.91	83.25	76.84	82.45
5	**AmelHPX8**	773	97.97	75.25	97.82	75.12		67.75	81.20	74.17	76.15	97.94	97.65	67.37	69.03	71.15	75.81	74.72	80.55	77.18
6	**AalHPX8**	782	67.99	68.64	67.91	67.74	67.75		66.67	67.77	68.81	67.84	67.59	87.41	66.68	67.06	67.14	67.95	67.83	68.02
7	**AchHPX8**	764	81.41	77.56	81.53	78.02	81.20	66.67		74.00	73.46	81.21	81.74	67.21	69.03	69.56	77.38	76.76	81.17	77.49
8	**AfaHPX8**	767	74.18	75.39	74.06	74.08	74.17	67.77	74.00		84.19	73.80	73.76	68.18	68.10	69.96	75.18	74.49	72.56	75.65
9	**AdiHPX8**	719	76.38	75.57	75.99	74.78	76.15	68.81	73.46	84.19		76.11	76.08	68.71	68.64	70.67	76.26	74.86	73.35	76.75
10	**AarHPX8**	768	98.43	75.09	98.00	74.93	97.94	67.84	81.21	73.80	76.11		98.00	67.58	69.20	71.31	75.89	74.27	80.24	76.98
11	**AmerHPX8**	753	97.61	75.35	97.90	75.18	97.65	67.59	81.74	73.76	76.08	98.00		67.04	69.06	71.54	76.01	74.69	80.53	76.81
12	**AdaHPX8**	803	67.50	69.03	67.51	68.43	67.37	87.41	67.21	68.18	68.71	67.58	67.04		65.66	66.24	67.49	69.38	67.71	67.77
13	**AsiHPX8**	768	69.45	69.19	69.30	69.31	69.03	66.68	69.03	68.10	68.64	69.20	69.06	65.66		78.31	68.88	68.13	69.70	69.00
14	**AatHPX8**	763	71.58	71.51	71.24	69.95	71.15	67.06	69.56	69.96	70.67	71.31	71.54	66.24	78.31		71.26	69.05	70.73	71.41
15	**AmaHPX8**	736	76.15	81.70	76.05	81.91	75.81	67.14	77.38	75.18	76.26	75.89	76.01	67.49	68.88	71.26		81.35	76.55	88.97
16	**AfuHPX8**	697	74.63	84.82	74.98	83.25	74.72	67.95	76.76	74.49	74.86	74.27	74.69	69.38	68.13	69.05	81.35		74.28	81.77
17	**AepiHPX8**	818	80.63	76.64	80.66	76.84	80.55	67.83	81.17	72.56	73.35	80.24	80.53	67.71	69.70	70.73	76.55	74.28		77.51
18	**AsHPX8**	714	77.43	82.24	77.52	82.45	77.18	68.02	77.49	75.65	76.75	76.98	76.81	67.77	69.00	71.41	88.97	81.77	77.51	

Conclusion of thesis

In this study, we identified and functionally characterized the role of two *A. stephensi* heme peroxidases - AsDBLOX and AsHPX8 in physiology and innate immunity. The AsDBLOX gene showed maximum expression in *A. stephensi* pupal stages, which indicated towards its crucial role in mosquito development. The AsDBLOX gene has two integrin binding motifs- RGD and LDV, along with two heme peroxidase domains (I and II), hence it is a double peroxinectin molecule. It is a unique gene also because, the HPX domain I lacks all features but substrate binding site which are characteristics of a heme peroxidase gene.

Further, phylogenetic analysis revealed that the DBLOX is an Ecdysozoa specific gene and uniquely, the African *Anopheles* malaria vectors i.e., the *A. gambiae* complex have an insertion of another integrin binding motif in addition to the two motifs present in all the worldwide distributed *Anopheles* vector species. Moreover, the two domains have different selection pressures acting on them. Hence, these findings led us to put forward a hypothesis, that DBLOX has undergone vigorous mutations in the course of evolution of the superfamily of heme peroxidases. Thus, we believe that this gene may diverge further and the HPX domain I may acquire other function in the course of evolution. Moreover, we found that AsDBLOX serves as an anti-bacterial gene in mosquito hemolymph compartment and its silencing increased the bacterial population. Uniquely, we observed that DBLOX was induced in the carcass of *P. berghei* infected females at a very early time point post infection (3 h). This was followed by an induction of TEP1 in infected midguts at 3 h. These data suggested that mosquito immunity is possibly elicited against *Plasmodium* parasite midgut stages, and hence further elucidation of immunity at this time point can be exploited for designing of experiments to achieve a transmission blocking vaccine.

The other heme peroxidase - AsHPX8 is a 2313bp long gene consisting of 3 exons. It is a true ortholog of AgHPX8. Its initial *in silico* analysis revealed that its regulatory region has binding sites for ecdysone, OVO, chorion factor and TGIF transcription factors. The AsHPX8 gene was also induced in sugar fed midguts of 5 day old *A. stephensi* female mosquitoes which corresponded to increased bacterial population. Moreover, the gene was induced in blood fed midguts as well as upon exogenous bacteria feeding. Hence, we propose that this gene may be playing a crucial role in maintaining midgut bacterial homeostasis. The AsHPX8 ortholog in *A. gambiae* was reported to be involved in oogenesis and possibly functions as a chorion peroxidase. Similarly, we observed that AsHPX8 gene was induced in blood fed carcasses. This time corresponds to mosquito egg development and thus we believe this gene functions as egg chorion peroxidase. Thus, we silenced this

gene; however, silencing had no effect on the number of eggs laid by the female mosquitoes. Surprisingly, we observed the induction of AsHPX7 gene which is the duplicated paralog of AsHPX8. This suggested that the redundancy in functioning of AsHPX8 gene induces its paralog ASHPX7, and counteracts the effects of gene silencing.

The study of the interactions between the mosquito immunity and its pathogens is not only interesting and equally challenging in terms of the fascinating cell biology but is also important for the development of novel approaches for prevention of malaria transmission. The mechanisms mosquito immunity has evolved to detect these pathogens are intricate and also provide us with novel targets for the development of strategies for prevention of malaria. Each of the malaria prevention methods requires a detailed understanding of the interactions between mosquitoes and *Plasmodium* parasite and this study will be helpful in designing transmission blocking strategies.

Future prospects

- Elucidation of innate immune responses from Indian malaria vector- *A. stephensi,* which differs significantly from African *A. gambiae* mosquito.
- The innate immunity of *Anopheles* mosquito against midgut stages of the *Plasmodium* parasite is least explored and further studies may help in selection of potential targets for transmission blocking vaccines.
- The mosquito midgut endosymbiotic bacteria play a crucial role against *Plasmodium* development and thus can be exploited for future transmission blocking strategies.
- Also, modulating the egg laying capacity of *Anopheles* mosquitoes may help in vector control measures.
- Pathogen binding studies using antibodies against AsDBLOX and AsHPX8 genes may give additional insights into their function.
- It would also be interesting to investigate the other HPX molecules in *A. stephensi* and study the crosstalk of these immune molecules in maintaining redox homeostasis and thus survivability of the mosquitoes.

Bibliography

Abraham, E.G., Pinto, S.B., Ghosh, A., Vanlandingham, D.L., Budd, A., Higgs, S., Kafatos, F.C., Jacobs-Lorena, M. & Michel, K. 2005. An immune-responsive serpin, SRPN6, mediates mosquito defense against malaria parasites. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 16327-16332.

Aguinaldo, A.M., Turbeville, J.M., Linford, L.S., Rivera, M.C., Garey, J.R., Raff, R.A. & Lake, J.A. 1997. Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature,* 387**,** 489-93.

Akagi, K. & Ueda, H. 2011. Regulatory mechanisms of ecdysone-inducible Blimp-1 encoding a transcriptional repressor that is important for the prepupal development in *Drosophila*. *Development, growth & differentiation,* 53**,** 697-703.

Akman-Anderson, L., Olivier, M. & Luckhart, S. 2007. Induction of nitric oxide synthase and activation of signaling proteins in *Anopheles* mosquitoes by the malaria pigment, hemozoin. *Infection and immunity,* 75**,** 4012-4019.

Albelda, S.M. & Buck, C.A. 1990. Integrins and other cell adhesion molecules. *The FASEB Journal,* 4**,** 2868-2880.

Amenya, D.A., Chou, W., Li, J., Yan, G., Gershon, P.D., James, A.A. & Marinotti, O. 2010. Proteomics reveals novel components of the *Anopheles gambiae* eggshell. *Journal of insect physiology,* 56**,** 1414-1419.

Amino, R., Giovannini, D., Thiberge, S., Gueirard, P., Boisson, B., Dubremetz, J.-F., Prévost, M.-C., Ishino, T., Yuda, M. & Ménard, R. 2008. Host cell traversal is important for progression of the malaria parasite through the dermis to the liver. *Cell host & microbe,* 3**,** 88-96.

Andersen, S.O. 2010a. Insect cuticular sclerotization: a review. *Insect biochemistry and molecular biology,* 40**,** 166-178.

Anvikar, A.R., Shah, N., Dhariwal, A.C., Sonal, G.S., Pradhan, M.M., Ghosh, S.K. & Valecha, N. 2016. Epidemiology of *Plasmodium vivax* malaria in India. *The American journal of tropical medicine and hygiene,* 95**,** 108-120.

Aoun, R.B., Hetru, C., Troxler, L., Doucet, D., Ferrandon, D. & Matt, N. 2011. Analysis of thioester-containing proteins during the innate immune response of *Drosophila melanogaster*. *Journal of innate immunity,* 3**,** 52-64.

Armistead, J.S., Morlais, I., Mathias, D.K., Jardim, J.G., Joy, J., Fridman, A., Finnefrock, A.C., Bagchi, A., Plebanski, M. & Scorpio, D.G. 2014. Antibodies to a single, conserved epitope in *Anopheles* APN1 inhibit universal transmission of *Plasmodium falciparum* and *Plasmodium vivax* malaria. *Infection and immunity,* 82**,** 818-829.

Ayyar, S., Jiang, J., Collu, A., White-Cooper, H. & White, R.A. 2003. *Drosophila* TGIF is essential for developmentally regulated transcription in spermatogenesis. *Development,* 130**,** 2841-2852.

Bailey, D., Basar, M.A., Nag, S., Bondhu, N., Teng, S. & Duttaroy, A. 2017a. The essential requirement of an animal heme peroxidase protein during the wing maturation process in *Drosophila*. *BMC developmental biology,* 17**,** 1.

Bartholomay, L.C., Farid, H.A., Ramzy, R.M. & Christensen, B.M. 2003. *Culex pipiens pipiens*: characterization of immune peptides and the influence of immune activation on development of *Wuchereria bancrofti*. *Molecular and biochemical parasitology,* 130**,** 43-50.

Bartholomay, L.C., Cho, W.-L., Rocheleau, T.A., Boyle, J.P., Beck, E.T., Fuchs, J.F., Liss, P., Rusch, M., Butler, K.M. & Wu, R.C.-C. 2004. Description of the transcriptomes of immune response-activated hemocytes from the mosquito vectors *Aedes aegypti* and *Armigeres subalbatus*. *Infection and immunity,* 72**,** 4114-4126.

Beisel, H.G., Kawabata, S.I., Iwanaga, S., Huber, R. & Bode, W. 1999. Tachylectin-2: crystal structure of a specific GlcNAc/GalNAc-binding lectin involved in the innate immunity host defense of the Japanese horseshoe crab *Tachypleus tridentatu*s. *The EMBO journal,* 18**,** 2313-2322.

Betteridge, D.J. 2000. What is oxidative stress? *Metabolism,* 49**,** 3-8.

Billker, O., Shaw, M., Margos, G. & Sinden, R. 1997. The roles of temperature, pH and mosquito factors as triggers of male and female gametogenesis of *Plasmodium berghe*i in vitro. *Parasitology,* 115**,** 1-7.

Blandin, S., Shiao, S.-H., Moita, L.F., Janse, C.J., Waters, A.P., Kafatos, F.C. & Levashina, E.A. 2004a. Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*. *Cell,* 116**,** 661-670.

Blandin, S.A. & Levashina, E.A. 2007. Phagocytosis in mosquito immune responses. *Immunological reviews,* 219**,** 8-16.

Bokharaei, H., Raz, A., Zakeri, S. & Djadid, N.D. 2012. 3'-RACE Amplification of aminopeptidase N gene from *Anopheles stephensi* applicable in transmission blocking vaccines. *Avicenna journal of medical biotechnology,* 4**,** 131.

Boman, H.G. & Steiner, H. 1981. Humoral immunity in *Cecropia* pupae. *Current topics in Microbiology and Immunology.* Springer.

Brandt, S.M., Jaramillo-Gutierrez, G., Kumar, S., Barillas-Mury, C. & Schneider, D.S. 2008. Use of a *Drosophila* model to identify genes regulating *Plasmodium* growth in the mosquito. *Genetics,* 180**,** 1671-1678.

Bryant, W.B. & Michel, K. 2014. Blood feeding induces hemocyte proliferation and activation in the African malaria mosquito, *Anopheles gambiae* Giles. *Journal of Experimental Biology,* 217**,** 1238-1245.

Buck, M., Nilsson, L.K., Brunius, C., Dabiré, R.K., Hopkins, R. & Terenius, O. 2016. Bacterial associations reveal spatial population dynamics in *Anopheles gambiae* mosquitoes. *Scientific reports,* 6**,** 22806.

Burge, C. & Karlin, S. 1997. Prediction of complete gene structures in human genomic DNA. *Journal of molecular biology,* 268**,** 78-94.

Burge, E.J., Burnett, L.E. & Burnett, K.G. 2009. Time-course analysis of peroxinectin mRNA in the shrimp *Litopenaeus vannamei* after challenge with *Vibrio campbellii*. *Fish Shellfish Immunol,* 27**,** 603-9.

Cai, S., Huang, Y., Wang, B., Jian, J. & Xu, Y. 2017. Tumor necrosis factor receptor-associated factor 6 (TRAF6) participates in peroxinectin gene expression in *Fenneropenaeus penicillatus*. *Fish Shellfish Immunol,* 64**,** 193-201.

Carter, R. 2001. Transmission blocking malaria vaccines. *Vaccine,* 19**,** 2309-2314.

Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M., Bayerlein, M. & Werner, T. 2005. MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics,* 21**,** 2933-2942.

Castillo, J., Robertson, A. & Strand, M. 2006. Characterization of hemocytes from the mosquitoes *Anopheles gambiae* and *Aedes aegypt*i. *Insect biochemistry and molecular biology,* 36**,** 891-903.

Chamary, J. & Hurst, L.D. 2005b. Evidence for selection on synonymous mutations affecting stability of mRNA secondary structure in mammals. *Genome biology,* 6**,** R75.

Chapman, A.D. 2009. Numbers of living species in Australia and the world.

Charman, S.A., Arbe-Barnes, S., Bathurst, I.C., Brun, R., Campbell, M., Charman, W.N., Chiu, F.C., Chollet, J., Craft, J.C. & Creek, D.J. 2011. Synthetic ozonide drug candidate OZ439 offers new hope for a single-dose cure of uncomplicated malaria. *Proceedings of the National Academy of Sciences,* 108**,** 4400-4405.

Charroux, B. & Royet, J. 2010. *Drosophila* immune response: From systemic antimicrobial peptide production in fat body cells to local defense in the intestinal tract. *Fly,* 4**,** 40-47.

Chaturvedi, N., Bharti, P.K., Tiwari, A. and Singh, N., 2015. Strategies & recent development of transmission-blocking vaccines against *Plasmodium falciparum*. The Indian journal of medical research, 143(6), p.696.

Chen, C.-H., Huang, H., Ward, C.M., Su, J.T., Schaeffer, L.V., Guo, M. & Hay, B.A. 2007. A synthetic maternal-effect selfish genetic element drives population replacement in *Drosophila*. *science,* 316**,** 597-600.

Chen, M.-S., Liu, S., Wang, H., Cheng, X., El Bouhssini, M. & Whitworth, R.J. 2016. Massive shift in gene expression during transitions between developmental stages of the gall midge, *Mayetiola Destructor*. *PloS one,* 11**,** e0155616.

Chen, S., Armistead, J.S., Provost-Javier, K.N., Sakamoto, J.M. & Rasgon, J.L. 2010. Duplication, concerted evolution and purifying selection drive the evolution of mosquito vitellogenin genes. *BMC evolutionary biology,* 10**,** 142.

Christensen, B.M., Huff, B.M., Miranpuri, G.S., Harris, K.L. & Christensen, L.A. 1989. Hemocyte population changes during the immune response of *Aedes aegypt*i to inoculated microfilariae of *Dirofilaria immitis*. *The Journal of parasitology***,** 119-123.

Cirimotich, C.M., Dong, Y., Garver, L.S., Sim, S. & Dimopoulos, G. 2010. Mosquito immune defenses against *Plasmodium* infection. *Developmental & Comparative Immunology,* 34**,** 387-395.

Cirimotich, C.M., Dong, Y., Clayton, A.M., Sandiford, S.L., Souza-Neto, J.A., Mulenga, M. & Dimopoulos, G. 2011. Natural microbe-mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*. *Science,* 332**,** 855-858.

Clayton, A.M., Cirimotich, C.M., Dong, Y. & Dimopoulos, G. 2013. Caudal is a negative regulator of the *Anopheles* IMD pathway that controls resistance to *Plasmodium falciparum* infection. *Developmental & Comparative Immunology,* 39**,** 323-332.

Clayton, A.M., Dong, Y. & Dimopoulos, G. 2014. The *Anopheles* innate immune system in the defense against malaria infection. *Journal of innate immunity,* 6**,** 169-181.

Clements, A.N. 1992. Development, nutrition and reproduction. *The biology of mosquitoes,* 1**,** 509.

Coletta, A., Pinney, J.W., Solís, D.Y.W., Marsh, J., Pettifer, S.R. & Attwood, T.K. 2010. Low-complexity regions within protein sequences have position-dependent roles. *BMC systems biology,* 4**,** 43.

Collins, F. & Paskewitz, S. 1996. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. *Insect molecular biology,* 5**,** 1-9.

Colovos, C. & Yeates, T. 1993. ERRAT: an empirical atom-based method for validating protein structures. *Protein Sci,* 2**,** 1511-1519.

Corby-Harris, V., Drexler, A., De Jong, L.W., Antonova, Y., Pakpour, N., Ziegler, R., Ramberg, F., Lewis, E.E., Brown, J.M. & Luckhart, S. 2010. Activation of Akt signaling reduces the prevalence and intensity of malaria parasite infection and lifespan in *Anopheles stephensi* mosquitoes. *PLoS pathogens,* 6**,** e1001003.

Cortés, A., Carret, C., Kaneko, O., Lim, B.Y.Y., Ivens, A. & Holder, A.A. 2007. Epigenetic silencing of *Plasmodium falciparum* genes linked to erythrocyte invasion. PLoS pathogens, 3, e107.

Crotti, E., Rizzi, A., Chouaia, B., Ricci, I., Favia, G., Alma, A., Sacchi, L., Bourtzis, K., Mandrioli, M., Cherif, A. and Bandi, C., 2010. Acetic acid bacteria, newly emerging symbionts of insects. *Applied and Environmental Microbiology*, 76(21), pp.6963-6970.

Da Silva, J.B., De Albuquerque, C.M., De Araujo, E.C., Peixoto, C.A. & Hurd, H. 2000. Immune defense mechanisms of *Culex quinquefasciatus* (Diptera: Culicidae) against *Candida albicans* infection. *Journal of Invertebrate Pathology,* 76**,** 257-262.

Das, S., Muleba, M., Stevenson, J.C., Pringle, J.C. & Norris, D.E. 2017. Beyond the entomological inoculation rate: characterizing multiple blood feeding behavior and *Plasmodium falciparum* multiplicity of infection in *Anopheles* mosquitoes in northern Zambia. *Parasites & vectors,* 10**,** 45.

De Almeida Oliveira, G., Lieberman, J. & Barillas-Mury, C. 2012. Epithelial nitration by a peroxidase/NOX5 system mediates mosquito antiplasmodial immunity. *Science,* 335**,** 856-859.

De O Gaio, A., Gusmão, D.S., Santos, A.V., Berbert-Molina, M.A., Pimenta, P.F. & Lemos, F.J. 2011. Contribution of midgut bacteria to blood digestion and egg production in *Aedes aegypti* (Diptera: Culicidae)(L.). *Parasites & vectors,* 4**,** 105.

Delves, M., Plouffe, D., Scheurer, C., Meister, S., Wittlin, S., Winzeler, E.A., Sinden, R.E. & Leroy, D. 2012. The activities of current antimalarial drugs on the life cycle stages of *Plasmodium*: a comparative study with human and rodent parasites. *PLoS medicine,* 9**,** e1001169.

Depristo, M.A., De Bakker, P.I., Lovell, S.C. & Blundell, T.L. 2003. *Ab initio* construction of polypeptide fragments: efficient generation of accurate, representative ensembles. *Proteins: Structure, Function, and Bioinformatics,* 51**,** 41-55.

Dev, V. & Sharma, V.P. 2013. The dominant mosquito vectors of human malaria in India. *Anopheles mosquitoes-New insights into malaria vectors.* InTech.

Devito, J.A. & Morris, S. 2003. Exploring the structure and function of the mycobacterial KatG protein using trans-dominant mutants. *Antimicrobial agents and chemotherapy,* 47**,** 188-195.

Dhawan, R., Gupta, K., Kajla, M., Kumar, S., Gakhar, S.K., Kakani, P., Choudhury, T.P. & Gupta, L. 2015. Molecular characterization of SOCS gene and its expression analysis on *Plasmodium berghei* infection in *Anopheles culicifacies*. *Acta Trop,* 152**,** 170-5.

Dhingra, N., Jha, P., Sharma, V.P., Cohen, A.A., Jotkar, R.M., Rodriguez, P.S., Bassani, D.G., Suraweera, W., Laxminarayan, R., Peto, R. & Million Death Study, C. 2010. Adult and child malaria mortality in India: a nationally representative mortality survey. *Lancet,* 376**,** 1768-74.

Dias, F.A., Gandara, A.C.P., Queiroz-Barros, F.G., Oliveira, R.L., Sorgine, M.H., Braz, G.R. & Oliveira, P.L. 2013. Ovarian dual oxidase (Duox) activity is essential for insect eggshell hardening and waterproofing. Journal of Biological Chemistry, 288, 35058-35067.

Dimopoulos, G., Richman, A., Müller, H.-M. & Kafatos, F.C. 1997. Molecular immune responses of the mosquito *Anopheles gambia*e to bacteria and malaria parasites. *Proceedings of the National Academy of Sciences,* 94**,** 11508-11513.

Dinglasan, R.R., Kalume, D.E., Kanzok, S.M., Ghosh, A.K., Muratova, O., Pandey, A. & Jacobs-Lorena, M. 2007. Disruption of *Plasmodium falciparum* development by antibodies against a conserved mosquito midgut antigen. *Proceedings of the National Academy of Sciences,* 104**,** 13461-13466.

Dong, Y., Aguilar, R., Xi, Z., Warr, E., Mongin, E. & Dimopoulos, G. 2006a. *Anopheles gambiae* immune responses to human and rodent *Plasmodium* parasite species. *PLoS Pathog,* 2**,** e52.

Dong, Y., Taylor, H.E. & Dimopoulos, G. 2006b. AgDscam, a hypervariable immunoglobulin domain-containing receptor of the *Anopheles gambiae* innate immune system. *PLoS biology,* 4**,** e229.

Dong, B., Liu, F., Gao, H., Wang, B. & Xiang, J. 2009a. cDNA cloning and gene expression pattern following bacterial challenge of peroxinectin in Chinese shrimp *Fenneropenaeus chinensis*. *Mol Biol Rep,* 36**,** 2333-9.

Dong, Y., Manfredini, F. & Dimopoulos, G. 2009b. Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS pathogens,* 5**,** e1000423.

Dong, C., Wei, Z. & Yang, G. 2011. Involvement of peroxinectin in the defence of red swamp crayfish *Procambarus clarkii* against pathogenic *Aeromonas hydrophila*. *Fish Shellfish Immunol,* 30**,** 1223-9.

Doron-Faigenboim, A., Stern, A., Mayrose, I., Bacharach, E. & Pupko, T. 2005. Selecton: a server for detecting evolutionary forces at a single amino-acid site. *Bioinformatics,* 21**,** 2101-2103.

Dostert, C., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D., Hetru, C., Hoffmann, J.A. & Imler, J.-L. 2005. The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of *Drosophila*. *Nature immunology,* 6**,** 946.

Drummond, D.A. & Wilke, C.O. 2008. Mistranslation-induced protein misfolding as a dominant constraint on coding-sequence evolution. *Cell,* 134**,** 341-352.

Du, Z.Q., Ren, Q., Huang, A.M., Fang, W.H., Zhou, J.F., Gao, L.J. & Li, X.C. 2013. A novel peroxinectin involved in antiviral and antibacterial immunity of mud crab, *Scylla paramamosain*. *Mol Biol Rep,* 40**,** 6873-81.

Duffy, P.E., Sahu, T., Akue, A., Milman, N. and Anderson, C., 2012. Pre-erythrocytic malaria vaccines: identifying the targets. *Expert review of vaccines*, 11(10), pp.1261-1280.

Dunford, H.B. 1999. *Heme peroxidases*, Wiley-vch.

Dunn, C.W., Hejnol, A., Matus, D.Q., Pang, K., Browne, W.E., Smith, S.A., Seaver, E., Rouse, G.W., Obst, M. & Edgecombe, G.D. 2008. Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature,* 452**,** 745.

Emami, S.N., Lindberg, B.G., Hua, S., Hill, S.R., Mozuraitis, R., Lehmann, P., Birgersson, G., Borg-Karlson, A.-K., Ignell, R. & Faye, I. 2017. A key malaria metabolite modulates vector blood seeking, feeding, and susceptibility to infection. *Science,* 355**,** 1076-1080.

Farrance, C.E., Rhee, A., Jones, R.M., Musiychuk, K., Shamloul, M., Sharma, S., Mett, V., Chichester, J.A., Streatfield, S.J. & Roeffen, W. 2011. A plant-produced Pfs230 vaccine candidate blocks transmission of *Plasmodium falciparum*. *Clinical and Vaccine Immunology,* 18**,** 1351-1357.

Ferguson, H.M. & Read, A.F. 2004. Mosquito appetite for blood is stimulated by *Plasmodium chabaudi* infections in themselves and their vertebrate hosts. *Malaria journal,* 3**,** 12.

Ferro, K., Ferro, D., Corra, F., Bakiu, R., Santovito, G. & Kurtz, J. 2017. Cu, Zn SOD genes in *Tribolium castaneum*: evolution, molecular characterisation and gene expression during immune priming. *Frontiers in Immunology,* 8**,** 1811.

Force, A., Lynch, M., Pickett, F.B., Amores, A., Yan, Y.-L. & Postlethwait, J. 1999. Preservation of duplicate genes by complementary, degenerative mutations. *Genetics,* 151**,** 1531-1545.

Fraiture, M., Baxter, R.H., Steinert, S., Chelliah, Y., Frolet, C., Quispe-Tintaya, W., Hoffmann, J.A., Blandin, S.A. & Levashina, E.A. 2009. Two mosquito LRR proteins function as complement control factors in the TEP1-mediated killing of *Plasmodium*. *Cell host & microbe,* 5**,** 273-284.

Frolet, C., Thoma, M., Blandin, S., Hoffmann, J.A. & Levashina, E.A. 2006. Boosting NF-κB-dependent basal immunity of *Anopheles gambiae* aborts development of *Plasmodium berghei*. *Immunity,* 25**,** 677-685.

Garcia, G.E., Wirtz, R.A., Barr, J.R., Woolfitt, A. & Rosenberg, R. 1998. Xanthurenic acid induces gametogenesis in *Plasmodium*, the malaria parasite. *Journal of Biological Chemistry,* 273**,** 12003-12005.

Geer, L.Y., Domrachev, M., Lipman, D.J. & Bryant, S.H. 2002. CDART: protein homology by domain architecture. *Genome research,* 12**,** 1619-1623.

Gendrin, M., Rodgers, F.H., Yerbanga, R.S., Ouédraogo, J.B., Basáñez, M.-G., Cohuet, A. & Christophides, G.K. 2015. Antibiotics in ingested human blood affect the mosquito microbiota and capacity to transmit malaria. *Nature communications,* 6**,** 5921.

Ghosh, A., Edwards, M. & Jacobs-Lorena, M. 2000. The journey of the malaria parasite in the mosquito: hopes for the new century. *Parasitology Today,* 16**,** 196-201.

Gilbert, L.I. 2004. Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*. *Molecular and cellular endocrinology,* 215**,** 1-10.

Gingold, H., Tehler, D., Christoffersen, N.R., Nielsen, M.M., Asmar, F., Kooistra, S.M., Christophersen, N.S., Christensen, L.L., Borre, M., Sorensen, K.D., Andersen, L.D., Andersen, C.L., Hulleman, E., Wurdinger, T., Ralfkiaer, E., Helin, K., Gronbaek, K., Orntoft, T., Waszak, S.M., Dahan, O., Pedersen, J.S., Lund, A.H. & Pilpel, Y. 2014. A dual program for translation regulation in cellular proliferation and differentiation. *Cell,* 158**,** 1281-92.

Gonzalez, M., Romestand, B., Fievet, J., Huvet, A., Lebart, M.-C., Gueguen, Y. & Bachère, E. 2005. Evidence in oyster of a plasma extracellular superoxide dismutase which binds LPS. *Biochemical and biophysical research communications,* 338**,** 1089-1097.

Gouagna, L.C., Mulder, B., Noubissi, E., Tchuinkam, T., Verhave, J.P. & Boudin, C. 1998. The early sporogonic cycle of *Plasmodium falciparum* in laboratory-infected *Anopheles gambiae*: an estimation of parasite efficacy. *Trop Med Int Health,* 3**,** 21-8.

Goujon, M., Mcwilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J. & Lopez, R. 2010. A new bioinformatics analysis tools framework at EMBL–EBI. *Nucleic acids research,* 38**,** W695-W699.

Gross, I., Georgel, P., Oertel-Buchheit, P., Schnarr, M. & Reichhart, J.-M. 1999. Dorsal-B, a splice variant of the *Drosophila* factor Dorsal, is a novel Rel/NF-κB transcriptional activator. *Gene,* 228**,** 233-242.

Gupta, L., Molina-Cruz, A., Kumar, S., Rodrigues, J., Dixit, R., Zamora, R.E. & Barillas-Mury, C. 2009. The STAT pathway mediates late-phase immunity against *Plasmodium* in the mosquito *Anopheles gambiae*. *Cell host & microbe,* 5**,** 498-507.

Gupta, L., Noh, J.Y., Jo, Y.H., Oh, S.H., Kumar, S., Noh, M.Y., Lee, Y.S., Cha, S.-J., Seo, S.J. & Kim, I. 2010. Apolipophorin-III mediates antiplasmodial epithelial responses in *Anopheles gambiae* (G3) mosquitoes. *PLoS One,* 5**,** e15410.

Häcker, U., Kaufmann, E., Hartmann, C., Jürgens, G., Knöchel, W. & Jäckle, H. 1995. The *Drosophila* fork head domain protein crocodile is required for the establishment of head structures. *The EMBO journal,* 14**,** 5306.

Hagedorn, H. 1989. Ecdysone Production by Insect Ovaries-a Citation Classic Commentary on the Ovary as a Source of Alpha-Ecdysone in an Adult Mosquito. *Current Contents:Agriculture Biology & Environmental Sciences***,** 14-14.

Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C., Katsanos, D., Gribble, M., Baker, D., Marois, E. & Russell, S. 2016. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nature biotechnology,* 34**,** 78.

Hayashi, M., Shinozuka, Y., Shigenobu, S., Sato, M., Sugimoto, M., Ito, S., Abe, K. & Kobayashi, S. 2017. Conserved role of Ovo in germline development in mouse and *Drosophila*. *Scientific reports,* 7**,** 40056.

Hernández-Martínez, S., Lanz, H., Rodrguez, M.H., González-Ceron, L. & Tsutsumi, V. 2002. Cellular-mediated reactions to foreign organisms inoculated into the hemocoel of *Anopheles albimanus* (Diptera: Culicidae). *Journal of medical entomology,* 39**,** 61-69.

Hershberg, R. & Petrov, D.A. 2008. Selection on codon bias. *Annu Rev Genet,* 42**,** 287-99.

Hetru, C. & Hoffmann, J.A. 2009. NF-κB in the immune response of *Drosophila*. *Cold Spring Harbor perspectives in biology,* 1**,** a000232.

Higgs, P.G. & Ran, W. 2008. Coevolution of codon usage and tRNA genes leads to alternative stable states of biased codon usage. *Molecular biology and evolution,* 25**,** 2279-2291.

Hill, A.V., 2011. Vaccines against malaria. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 366(1579), pp.2806-2814.

Hillyer, J.F., Barreau, C. & Vernick, K.D. 2007. Efficiency of salivary gland invasion by malaria sporozoites is controlled by rapid sporozoite destruction in the mosquito haemocoel. *International journal for parasitology,* 37**,** 673-681.

Hillyer, J.F., Schmidt, S.L. & Christensen, B.M. 2003a. Hemocyte-mediated phagocytosis and melanization in the mosquito *Armigeres subalbatus* following immune challenge by bacteria. *Cell and tissue research,* 313**,** 117-127.

Hillyer, J.F., Schmidt, S.L. & Christensen, B.M. 2003b. Rapid phagocytosis and melanization of bacteria and *Plasmodium* sporozoites by hemocytes of the mosquito *Aedes aegypt*i. *Journal of Parasitology,* 89**,** 62-69.

Hillyer, J.F., Schmidt, S.L. & Christensen, B.M. 2004. The antibacterial innate immune response by the mosquito *Aedes aegypti* is mediated by hemocytes and independent of Gram type and pathogenicity. *Microbes and infection,* 6**,** 448-459.

Hillyer, J.F. & Strand, M.R. 2014. Mosquito hemocyte-mediated immune responses. *Current opinion in insect science,* 3**,** 14-21.

Hoffmann, J.A., Kafatos, F.C., Janeway, C.A. & Ezekowitz, R.A. 1999. Phylogenetic perspectives in innate immunity. *Science,* 284**,** 1313-8.

Holmblad, T. & Söderhäll, K. 1999. Cell adhesion molecules and antioxidative enzymes in a crustacean, possible role in immunity. *Aquaculture,* 172**,** 111-123.

Hsu, P.I., Liu, C.H., Tseng, D.Y., Lee, P.P. & Cheng, W. 2006. Molecular cloning and characterisation of peroxinectin, a cell adhesion molecule, from the giant freshwater prawn *Macrobrachium rosenbergii*. *Fish Shellfish Immunol,* 21**,** 1-10.

Hughes, A.L. 2012. Evolution of the Heme Peroxidases of Culicidae (Diptera). *Psyche: A Journal of Entomology,* 2012.

Hurst, L.D. 2002. The Ka/Ks ratio: diagnosing the form of sequence evolution. *TRENDS in Genetics,* 18**,** 486-487.

Iatsenko, I., Kondo, S., Mengin-Lecreulx, D. & Lemaitre, B. 2016. PGRP-SD, an extracellular pattern-recognition receptor, enhances peptidoglycan-mediated activation of the *Drosophila* Imd Pathway. *Immunity,* 45**,** 1013-1023.

Ikemura, T. 1981. Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes: a proposal for a synonymous codon choice that is optimal for the *E. coli* translational system. *Journal of molecular biology,* 151**,** 389-409.

Isaacs, A.T., Li, F., Jasinskiene, N., Chen, X., Nirmala, X., Marinotti, O., Vinetz, J.M. & James, A.A. 2011. Engineered resistance to *Plasmodium falciparu*m development in transgenic *Anopheles stephensi*. *PLoS pathogens,* 7**,** e1002017.

Ishii, K., Hamamoto, H., Kamimura, M. & Sekimizu, K. 2008. Activation of the silkworm cytokine by bacterial and fungal cell wall components via a reactive oxygen species-triggered mechanism. *Journal of Biological Chemistry,* 283**,** 2185-2191.

Ito, J., Ghosh, A., Moreira, L.A., Wimmer, E.A. & Jacobs-Lorena, M. 2002. Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature,* 417**,** 452.

Janeway, C.A., Jr. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol,* 54 Pt 1**,** 1-13.

Jia, Z., Zhang, T., Jiang, S., Wang, M., Cheng, Q., Sun, M., Wang, L. & Song, L. 2015. An integrin from oyster *Crassostrea gigas* mediates the phagocytosis toward Vibrio splendidus through LPS binding activity. *Developmental & Comparative Immunology,* 53**,** 253-264.

Jiang, J., Zhou, Z., Dong, Y., Zhao, Z., Sun, H., Wang, B., Jiang, B., Chen, Z. & Gao, S. 2018. Comparative expression analysis of immune-related factors in the sea cucumber *Apostichopus japonicus*. *Fish & shellfish immunology,* 72**,** 342-347.

Jiggins, F.M. & Hurst, G.D. 2003. The evolution of parasite recognition genes in the innate immune system: purifying selection on *Drosophila melanogaster* peptidoglycan recognition proteins. *Journal of molecular evolution,* 57**,** 598-605.

Johansson, M.W. 1999. Cell adhesion molecules in invertebrate immunity. *Developmental & Comparative Immunology,* 23**,** 303-315.

Johansson, M.W., Holmblad, T., Thornqvist, P.-O., Cammarata, M., Parrinello, N. & Soderhall, K. 1999a. A cell-surface superoxide dismutase is a binding protein for peroxinectin, a cell-adhesive peroxidase in crayfish. *Journal of Cell Science,* 112**,** 917-925.

Johansson, M.W., Lind, M.I., Holmblad, T., Thornqvist, P.-O. & Soderhall, K. 1995a. Peroxinectin, a novel cell adhesion protein from crayfish blood. *Biochemical and biophysical research communications,* 216**,** 1079-1087.

Johansson, M.W. & Söderhäll, K. 1988. Isolation and purification of a cell adhesion factor from crayfish blood cells. *The Journal of cell biology,* 106**,** 1795-1803.

Johansson, M.W. & Söderhäll, K. 1989. A cell adhesion factor from crayfish haemocytes has degranulating activity towards crayfish granular cells. *Insect biochemistry,* 19**,** 183-190.

Jünger, M.A., Rintelen, F., Stocker, H., Wasserman, J.D., Végh, M., Radimerski, T., Greenberg, M.E. & Hafen, E. 2003. The *Drosophila* forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. *Journal of biology,* 2**,** 20.

Kajla, M., Choudhury, T.P., Kakani, P., Gupta, K., Dhawan, R., Gupta, L. & Kumar, S. 2016a. Silencing of *Anopheles stephensi* heme peroxidase HPX15 activates diverse immune pathways to regulate the growth of midgut bacteria. *Frontiers in microbiology,* 7.

Kajla, M., Gupta, K., Gupta, L. & Kumar, S. 2015b. A fine-tuned management between physiology and immunity maintains the gut microbiota in insects. *Biochem. Physiol,* 4**,** 10.4172.

Kajla, M., Gupta, K., Kakani, P., Dhawan, R., Choudhury, T.P., Gupta, L., Gakhar, S.K. & Kumar, S. 2015c. Identification of an *Anopheles* lineage-specific unique heme peroxidase HPX15: a plausible candidate for arresting malaria parasite development. *J. Phylogenetics Evol. Biol,* 3**,** 10.4172.

Kajla, M., Kakani, P., Choudhury, T.P., Gupta, K., Gupta, L. & Kumar, S. 2016b. Characterization and expression analysis of gene encoding heme peroxidase HPX15 in major Indian malaria vector *Anopheles stephensi* (Diptera: Culicidae). *Acta tropica,* 158**,** 107-116.

Kajla, M., Parik Kakani, T.P.C., Kumar, V., Gupta, K., Dhawan, R., Gupta, L. & Kumar, S. 2017. *Anopheles stephensi* heme peroxidase HPX15 suppresses midgut immunity to support *Plasmodium* development. *Frontiers in Immunology,* 8.

Källberg, M., Margaryan, G., Wang, S., Ma, J. & Xu, J. 2014. RaptorX server: a resource for template-based protein structure modeling. *Protein Structure Prediction***,** 17-27.

Källberg, M., Wang, H., Wang, S., Peng, J., Wang, Z., Lu, H. & Xu, J. 2012. Template-based protein structure modeling using the RaptorX web server. *Nature protocols,* 7**,** 1511-1522.

Kaslow, D.C. 2002. Transmission-blocking vaccines. *Malaria Immunology.* Karger Publishers.

Kawabata, S.-I., Nagayama, R., Hirata, M., Shigenaga, T., Agarwala, K.L., Saito, T., Cho, J., Nakajima, H., Takagi, T. & Iwanaga, S. 1996. Tachycitin, a small granular component in horseshoe crab hemocytes, is an antimicrobial protein with chitin-binding activity. *The Journal of Biochemistry,* 120**,** 1253-1260.

Kawamoto, F., Alejo-Blanco, R., Fleck, S.L. & Sinden, R.E. 1991. *Plasmodium berghei*: ionic regulation and the induction of gametogenesis. *Experimental parasitology,* 72**,** 33-42.

Kelley, L.A. & Sternberg, M.J. 2009. Protein structure prediction on the Web: a case study using the Phyre server. *Nature protocols,* 4**,** 363-371.

King, J.G. & Hillyer, J.F. 2012. Infection-induced interaction between the mosquito circulatory and immune systems. *PLoS pathogens,* 8**,** e1003058.

King, J.G. & Hillyer, J.F. 2013. Spatial and temporal in vivo analysis of circulating and sessile immune cells in mosquitoes: hemocyte mitosis following infection. *BMC biology,* 11**,** 55.

Klebanoff, S.J. 1968. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. *Journal of bacteriology,* 95**,** 2131-2138.

Kleino, A., Valanne, S., Ulvila, J., Kallio, J., Myllymäki, H., Enwald, H., Stöven, S., Poidevin, M., Ueda, R. & Hultmark, D. 2005. Inhibitor of apoptosis 2 and TAK1-binding protein are components of the *Drosophila* Imd pathway. *The EMBO journal,* 24**,** 3423-3434.

Klowden, M.J. 2007. Making generalizations about vectors: Is there a physiology of "the mosquito"? *Entomological Research,* 37**,** 1-13.

Kobayashi, M., Johansson, M.W. & Söderhäll, K. 1990. The 76 kD cell-adhesion factor from crayfish haemocytes promotes encapsulation in vitro. *Cell and Tissue Research,* 260**,** 13-18.

Koella, J.C., Sörensen, F.L. & Anderson, R. 1998. The malaria parasite, *Plasmodium falciparum*, increases the frequency of multiple feeding of its mosquito vector, *Anopheles gambiae*. *Proceedings of the Royal Society of London B: Biological Sciences,* 265**,** 763-768.

Konstandi, O.A., Papassideri, I.S., Stravopodis, D.J., Kenoutis, C.A., Hasan, Z., Katsorchis, T., Wever, R. & Margaritis, L.H. 2005. The enzymatic component of *Drosophila melanogaster* chorion is the Pxd peroxidase. *Insect biochemistry and molecular biology,* 35**,** 1043-1057.

Koonin, E.V. & Rogozin, I.B. 2003. Getting positive about selection. BioMed Central.

Korochkina, S., Barreau, C., Pradel, G., Jeffery, E., Li, J., Natarajan, R., Shabanowitz, J., Hunt, D., Frevert, U. & Vernick, K.D. 2006. A mosquito-specific protein family includes candidate receptors for malaria sporozoite invasion of salivary glands. *Cellular microbiology,* 8**,** 163-175.

Krieger, E., Joo, K., Lee, J., Lee, J., Raman, S., Thompson, J., Tyka, M., Baker, D. & Karplus, K. 2009. Improving physical realism, stereochemistry, and side-chain accuracy in homology modeling: four approaches that performed well in CASP8. *Proteins: Structure, Function, and Bioinformatics,* 77**,** 114-122.

Krzywinski, J. & Besansky, N.J. 2003. Molecular systematics of *Anopheles*: from subgenera to subpopulations. *Annual Review of Entomology,* 48**,** 111-139.

Krzywinski, J., Wilkerson, R.C. & Besansky, N.J. 2001. Toward understanding Anophelinae (Diptera, Culicidae) phylogeny: insights from nuclear single-copy genes and the weight of evidence. *Systematic Biology,* 50**,** 540-556.

Kumar, S., Gupta, L., Han, Y.S. & Barillas-Mury, C. 2004. Inducible peroxidases mediate nitration of anopheles midgut cells undergoing apoptosis in response to *Plasmodium* invasion. *Journal of Biological Chemistry,* 279**,** 53475-53482.

Kumar, S., Molina-Cruz, A., Gupta, L., Rodrigues, J. & Barillas-Mury, C. 2010a. A peroxidase/dual oxidase system modulates midgut epithelial immunity in *Anopheles gambiae*. *Science,* 327**,** 1644-8.

Kumar, S., Molina-Cruz, A., Gupta, L., Rodrigues, J. & Barillas-Mury, C. 2010b. A peroxidase/dual oxidase system modulates midgut epithelial immunity in *Anopheles gambiae*. *Science,* 327**,** 1644-1648.

Kumar, S., Stecher, G. & Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular biology and evolution,* 33**,** 1870-1874.

Kumaratilake, L.M. & Ferrante, A. 2000. Opsonization and phagocytosis of *Plasmodium falciparum* merozoites measured by flow cytometry. *Clinical and diagnostic laboratory immunology,* 7**,** 9-13.

Lagueux, M., Perrodou, E., Levashina, E.A., Capovilla, M. & Hoffmann, J.A. 2000. Constitutive expression of a complement-like protein in toll and JAK gain-of-function mutants of *Drosophila*. *Proceedings of the National Academy of Sciences,* 97**,** 11427-11432.

Lai, S.-C., Chen, C.-C. & Hou, R.F. 2001. Electron microscopic observations on wound-healing in larvae of the mosquito *Armigeres subalbatus* (Diptera: Culicidae). *Journal of medical entomology,* 38**,** 836-843.

Lavazec, C., Boudin, C., Lacroix, R., Bonnet, S., Diop, A., Thiberge, S., Boisson, B., Tahar, R. & Bourgouin, C. 2007. Carboxypeptidases B of *Anopheles gambiae* as targets for a *Plasmodium falciparum* transmission-blocking vaccine. *Infection and immunity,* 75**,** 1635-1642.

League, G.P., Estévez-Lao, T.Y., Yan, Y., Garcia-Lopez, V.A. & Hillyer, J.F. 2017. *Anopheles gambiae* larvae mount stronger immune responses against bacterial infection than adults: evidence of adaptive decoupling in mosquitoes. *Parasites & vectors,* 10**,** 367.

Lehane, A.M., Saliba, K.J., Allen, R.J. and Kirk, K., 2004. Choline uptake into the malaria parasite is energized by the membrane potential. *Biochemical and biophysical research* communications, 320(2), pp.311-317.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.-M. & Hoffmann, J.A. 1996. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell,* 86**,** 973-983.

Lemaitre, B. & Hoffmann, J. 2007. The host defense of *Drosophila melanogaste*r. *Annu. Rev. Immunol.,* 25**,** 697-743.

Lenaerts, C., Cools, D., Verdonck, R., Verbakel, L., Broeck, J.V. & Marchal, E. 2017. The ecdysis triggering hormone system is essential for successful moulting of a major hemimetabolous pest insect, *Schistocerca gregaria*. *Scientific Reports,* 7.

Letunic, I. & Bork, P. 2017. 20 years of the SMART protein domain annotation resource. *Nucleic acids research,* 46**,** D493-D496.

Leulier, F. & Royet, J. 2009. Maintaining immune homeostasis in fly gut. *Nature immunology,* 10**,** 936.

Levashina, E.A. 2004. Immune responses in *Anopheles gambiae*. *Insect Biochemistry and Molecular Biology,* 34**,** 673-678.

Levashina, E.A., Moita, L.F., Blandin, S., Vriend, G., Lagueux, M. & Kafatos, F.C. 2001a. Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*. *Cell,* 104**,** 709-718.

Lewis, P.W., Beall, E.L., Fleischer, T.C., Georlette, D., Link, A.J. & Botchan, M.R. 2004. Identification of a *Drosophila* Myb-E2F2/RBF transcriptional repressor complex. *Genes & development,* 18**,** 2929-2940.

Li, J. 1994. Egg chorion tanning in *Aedes aegypt*i mosquito. *Comparative Biochemistry and Physiology Part A: Physiology,* 109**,** 835-843.

Li, J., Hodgeman, B.A. & Christensen, B.M. 1996. Involvement of peroxidase in chorion hardening in *Aedes aegypti*. *Insect biochemistry and molecular biology,* 26**,** 309-317.

Li, J.J. & Biggin, M.D. 2015. Statistics requantitates the central dogma. *Science,* 347**,** 1066-1067.

Li, J.S. & Li, J. 2005. Characterization of N-linked oligosaccharides in chorion peroxidase of *Aedes aegypti* mosquito. *Protein Sci,* 14**,** 2370-86.

Li, J.S. & Li, J. 2006. Major chorion proteins and their crosslinking during chorion hardening in *Aedes aegypti* mosquitoes. *Insect biochemistry and molecular biology,* 36**,** 954-964.

Liang, Z., Lindblad, P., Beauvais, A., Johansson, M.W., Latgé, J.-P., Hall, M., Cerenius, L. & Söderhäll, K. 1992. Crayfish α-macroglobulin and 76 kDa protein; their biosynthesis and subcellular localization of the 76 kDa protein. *Journal of insect physiology,* 38**,** 987-995.

Lin, X., Cerenius, L., Lee, B.L. & Söderhäll, K. 2007. Purification of properoxinectin, a myeloperoxidase homologue and its activation to a cell adhesion molecule. *Biochimica et Biophysica Acta (BBA)-General Subjects,* 1770**,** 87-93.

Lin, Y.C., Vaseeharan, B. & Chen, J.C. 2010. Molecular cloning of mud crab Scylla serrata peroxinectin and its expression following *Vibrio alginolyticus* and peptidoglycan injections. *Fish Shellfish Immunol,* 28**,** 205-11.

Liu, B., Zheng, Y., Yin, F., Yu, J., Silverman, N. & Pan, D. 2016. Toll receptor-mediated Hippo signaling controls innate immunity in *Drosophila*. *Cell,* 164**,** 406-419.

Liu, C.-H., Cheng, W., Kuo, C.-M. & Chen, J.-C. 2004. Molecular cloning and characterisation of a cell adhesion molecule, peroxinectin from the white shrimp *Litopenaeus vannamei. Fish & shellfish immunology,* 17**,** 13-26.

Liu, C.H., Cheng, W. & Chen, J.C. 2005. The peroxinectin of white shrimp *Litopenaeus vannamei* is synthesised in the semi-granular and granular cells, and its transcription is up-regulated with *Vibrio alginolyticu*s infection. *Fish Shellfish Immunol,* 18**,** 431-44.

Liu, C.H., Yeh, S.P., Hsu, P.Y. & Cheng, W. 2007. Peroxinectin gene transcription of the giant freshwater prawn *Macrobrachium rosenbergii* under intrinsic, immunostimulant, and chemotherapeutant influences. *Fish Shellfish Immunol,* 22**,** 408-17.

Livak, K.J. & Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods,* 25**,** 402-408.

Lombardo, F., Ghani, Y., Kafatos, F.C. & Christophides, G.K. 2013. Comprehensive genetic dissection of the hemocyte immune response in the malaria mosquito *Anopheles gambia*e. *PLoS pathogens,* 9**,** e1003145.

Lowenberger, C., Charlet, M., Vizioli, J., Kamal, S., Richman, A., Christensen, B.M. & Bulet, P. 1999. Antimicrobial activity spectrum, cDNA cloning, and mRNA expression of a newly isolated member of the cecropin family from the mosquito vector *Aedes aegypti*. *Journal of Biological Chemistry,* 274**,** 20092-20097.

Lu, A., Zhang, Q., Zhang, J., Yang, B., Wu, K., Xie, W., Luan, Y.-X. & Ling, E. 2014. Insect prophenoloxidase: the view beyond immunity. *Frontiers in physiology,* 5.

Luckhart, S., Vodovotz, Y., Cui, L. & Rosenberg, R. 1998. The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. *Proceedings of the National Academy of Sciences,* 95**,** 5700-5705.

Luckhart, S., Crampton, A.L., Zamora, R., Lieber, M.J., Dos Santos, P.C., Peterson, T.M., Emmith, N., Lim, J., Wink, D.A. & Vodovotz, Y. 2003. Mammalian transforming growth factor β1 activated after ingestion by *Anopheles stephensi* modulates mosquito immunity. *Infection and immunity,* 71**,** 3000-3009.

Lv, S., Lu, B., Xu, J., Xu, H., Zhao, J., Li, S., Li, Y. & Chen, Y. 2015. Immune response of peroxinectin of Chinese mitten crab *Eriocheir sinensis* to exterior stimulation. *Dev Comp Immunol,* 51**,** 56-64.

Lynch, M. & Conery, J.S. 2000. The evolutionary fate and consequences of duplicate genes. *Science,* 290**,** 1151-1155.

Mace, K.A., Pearson, J.C. & Mcginnis, W. 2005. An epidermal barrier wound repair pathway in *Drosophila* is mediated by grainy head. *Science,* 308**,** 381-385.

Majello, B., Kenyon, L.C. & Dalla-Favera, R. 1986. Human c-myb protooncogene: nucleotide sequence of cDNA and organization of the genomic locus. *Proceedings of the National Academy of Sciences,* 83**,** 9636-9640.

Marchler-Bauer, A., Derbyshire, M.K., Gonzales, N.R., Lu, S., Chitsaz, F., Geer, L.Y., Geer, R.C., He, J., Gwadz, M. & Hurwitz, D.I. 2014. CDD: NCBI's conserved domain database. *Nucleic acids research,* 43**,** D222-D226.

Marinotti, O., Calvo, E., Nguyen, Q.K., Dissanayake, S., Ribeiro, J. & James, A.A. 2006. Genome-wide analysis of gene expression in adult *Anopheles gambia*e. *Insect molecular biology,* 15**,** 1-12.

Marrelli, M.T., Li, C., Rasgon, J.L. & Jacobs-Lorena, M. 2007. Transgenic malaria-resistant mosquitoes have a fitness advantage when feeding on *Plasmodium*-infected blood. *Proceedings of the National Academy of Sciences,* 104**,** 5580-5583.

Mathelier, A., Zhao, X., Zhang, A.W., Parcy, F., Worsley-Hunt, R., Arenillas, D.J., Buchman, S., Chen, C.-Y., Chou, A. & Ienasescu, H. 2013. JASPAR 2014: an extensively expanded and updated open-access database of transcription factor binding profiles. *Nucleic acids research,* 42**,** D142-D147.

Mathias, D., Plieskatt, J., Armistead, J., Bethony, J.M., Abdul-Majid, K., Mcmillan, A., Angov, E., Aryee, M., Zhan, B. & Gillespie, P. 2012. Expression, immunogenicity, histopathology, and potency of a mosquito-based malaria transmission-blocking recombinant vaccine. *Infection and immunity,* 80**,** 1606-1614.

Mcbride, C.S. 2007. Rapid evolution of smell and taste receptor genes during host specialization in *Drosophila sechellia*. *Proceedings of the National Academy of Sciences,* 104**,** 4996-5001.

Medica, D.L. & Sinnis, P. 2005. Quantitative dynamics of *Plasmodium yoelii* sporozoite transmission by infected anopheline mosquitoes. *Infection and immunity,* 73**,** 4363-4369.

Medzhitov, R., Preston-Hurlburt, P. & Janeway, C.A., Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature,* 388**,** 394-7.

Meister, S., Kanzok, S.M., Zheng, X.-L., Luna, C., Li, T.-R., Hoa, N.T., Clayton, J.R., White, K.P., Kafatos, F.C. & Christophides, G.K. 2005. Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito *Anopheles gambiae*. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 11420-11425.

Mevel-Ninio, M., Terracol, R. & Kafatos, F. 1991. The ovo gene of *Drosophila* encodes a zinc finger protein required for female germ line development. *The EMBO journal,* 10**,** 2259.

Meyer, A. & Zardoya, R. 2003. Recent advances in the (molecular) phylogeny of vertebrates. *Annual Review of Ecology, Evolution, and Systematics,* 34**,** 311-338.

Minard, G., Mavingui, P. & Moro, C.V. 2013. Diversity and function of bacterial microbiota in the mosquito holobiont. *Parasites & vectors,* 6**,** 146.

Moita, L.F., Wang-Sattler, R., Michel, K., Zimmermann, T., Blandin, S., Levashina, E.A. & Kafatos, F.C. 2005. *In vivo* identification of novel regulators and conserved pathways of phagocytosis in *A. gambiae*. *Immunity,* 23**,** 65-73.

Montoya-Burgos, J.I. 2011. Patterns of positive selection and neutral evolution in the protein-coding genes of Tetraodon and Takifugu. *PLoS One,* 6**,** e24800.

Moskalyk, L., Oo, M. & Jacobs-Lorena, M. 1996. Peritrophic matrix proteins of *Anopheles gambiae* and *Aedes aegypti*. *Insect molecular biology,* 5**,** 261-268.

Murzin, A.G., Brenner, S.E., Hubbard, T. & Chothia, C. 1995. SCOP: a structural classification of proteins database for the investigation of sequences and structures. *Journal of molecular biology,* 247**,** 536-540.

Mussabekova, A., Daeffler, L. & Imler, J.-L. 2017. Innate and intrinsic antiviral immunity in *Drosophila*. *Cellular and Molecular Life Sciences***,** 1-16.

Muyskens, J.B. & Guillemin, K. 2008. Bugs inside Bugs: what the fruit fly can teach us about immune and microbial balance in the gut. *Cell host & microbe,* 3**,** 117-118.

Nakatogawa, S.-I., Oda, Y., Kamiya, M., Kamijima, T., Aizawa, T., Clark, K.D., Demura, M., Kawano, K., Strand, M.R. & Hayakawa, Y. 2009. A novel peptide mediates aggregation and migration of hemocytes from an insect. *Current Biology,* 19**,** 779-785.

Nakhleh, J., El Moussawi, L. & Osta, M.A. 2017. Chapter Three-The Melanization Response in Insect Immunity. *Advances in Insect Physiology,* 52**,** 83-109.

Nappi, A.J. & Ottaviani, E. 2000. Cytotoxicity and cytotoxic molecules in invertebrates. *Bioessays,* 22**,** 469-480.

Neafsey, D.E., Waterhouse, R.M., Abai, M.R., Aganezov, S.S., Alekseyev, M.A., Allen, J.E., Amon, J., Arcà, B., Arensburger, P. & Artemov, G. 2015. Highly evolvable malaria vectors: the genomes of 16 *Anopheles* mosquitoes. *Science,* 347**,** 1258522.

Nei, M. & Rooney, A.P. 2005. Concerted and birth-and-death evolution of multigene families. *Annu. Rev. Genet.,* 39**,** 121-152.

Ng Welinder, K.G. 1991. Bacterial catalase-peroxidases are gene duplicated members of the plant peroxidase superfamily. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology,* 1080**,** 215-220.

Ngwa, C., Glöckner, V., Abdelmohsen, U.R., Scheuermayer, M., Fischer, R., Hentschel, U. & Pradel, G. 2013. 16S rRNA gene-based identification of *Elizabethkingia meningoseptica* (Flavobacteriales: Flavobacteriaceae) as a dominant midgut bacterium of the Asian malaria vector *Anopheles stephensi* (Dipteria: Culicidae) with antimicrobial activities. *Journal of medical entomology,* 50**,** 404-414.

Nielsen, R. & Yang, Z. 1998. Likelihood models for detecting positively selected amino acid sites and applications to the HIV-1 envelope gene. *Genetics,* 148**,** 929-936.

Nishihara, H., Maruyama, S. & Okada, N. 2009. Retroposon analysis and recent geological data suggest near-simultaneous divergence of the three superorders of mammals. *Proceedings of the National Academy of Sciences,* 106**,** 5235-5240.

Nunez-Acuna, G. & Gallardo-Escarate, C. 2015. Two novel male-associated peroxinectin genes are downregulated by exposure to delousing drugs in *Caligus rogercresseyi*. *Gene,* 557**,** 98-102.

Nwane, P., Etang, J., Chouaïbou, M., Toto, J.C., Koffi, A., Mimpfoundi, R. & Simard, F. 2013. Multiple insecticide resistance mechanisms in *Anopheles gambiae* sl populations from Cameroon, Central Africa. *Parasites & vectors,* 6**,** 41.

Ohno, S. Gene duplication and the uniqueness of vertebrate genomes circa 1970–1999. Seminars in cell & developmental biology, 1999. Elsevier, 517-522.

Okulate, M., Kalume, D., Reddy, R., Kristiansen, T., Bhattacharyya, M., Chaerkady, R., Pandey, A. & Kumar, N. 2007. Identification and molecular characterization of a novel protein Saglin as a target of monoclonal antibodies affecting salivary gland infectivity of *Plasmodium* sporozoites. *Insect molecular biology,* 16**,** 711-722.

Oliveira, J.H.M., Gonçalves, R.L., Lara, F.A., Dias, F.A., Gandara, A.C.P., Menna-Barreto, R.F., Edwards, M.C., Laurindo, F.R., Silva-Neto, M.A. & Sorgine, M.H. 2011. Blood meal-derived heme decreases ROS levels in the midgut of *Aedes aegypti* and allows proliferation of intestinal microbiota. *PLoS pathogens,* 7**,** e1001320.

Oliver, B., Perrimon, N. & Mahowald, A. 1987. The ovo locus is required for sex-specific germ line maintenance in *Drosophila*. *Genes & Development,* 1**,** 913-923.

Owusu-Ansah, E. & Banerjee, U. 2009. Reactive oxygen species prime *Drosophila* haematopoietic progenitors for differentiation. *Nature,* 461**,** 537.

Pace, C.N. & Scholtz, J.M. 1998. A helix propensity scale based on experimental studies of peptides and proteins. *Biophys J,* 75**,** 422-7.

Pal, S. & Wu, L.P. 2009. Lessons from the fly: pattern recognition in *Drosophila melanogaster*. *Target Pattern Recognition in Innate Immunity.* Springer.

Pancer, Z. & Cooper, M.D. 2006. The evolution of adaptive immunity. *Annu. Rev. Immunol.,* 24**,** 497-518.

Park, J., Stanley, D. & Kim, Y. 2014. Roles of peroxinectin in PGE2-mediated cellular immunity in *Spodoptera exigua*. *PLoS One,* 9**,** e105717.

Passardi, F., Theiler, G., Zamocky, M., Cosio, C., Rouhier, N., Teixera, F., Margis-Pinheiro, M., Ioannidis, V., Penel, C. & Falquet, L. 2007. PeroxiBase: the peroxidase database. *Phytochemistry,* 68**,** 1605-1611.

Patient, R.K. & Mcghee, J.D. 2002. The GATA family (vertebrates and invertebrates). *Current opinion in genetics & development,* 12**,** 416-422.

Pearson, W.R. 2013. An introduction to sequence similarity ("homology") searching. *Curr Protoc Bioinformatics,* Chapter 3**,** Unit3 1.

Pearson, W.R. 2014. BLAST and FASTA similarity searching for multiple sequence alignment. *Methods Mol Biol,* 1079**,** 75-101.

Pechmann, S. & Frydman, J. 2013. Evolutionary conservation of codon optimality reveals hidden signatures of cotranslational folding. *Nat Struct Mol Biol,* 20**,** 237-43.

Peterfi, Z. & Geiszt, M. 2014. Peroxidasins: novel players in tissue genesis. *Trends Biochem Sci,* 39**,** 305-7.

Péterfi, Z. & Geiszt, M. 2014. Peroxidasins: novel players in tissue genesis. *Trends in biochemical sciences,* 39**,** 305-307.

Petersen, T.N., Brunak, S., Von Heijne, G. & Nielsen, H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature methods,* 8**,** 785-786.

Phillips, D.R. & Clark, K.D. 2017. *Bombyx mori* and *Aedes aegypti* form multi-functional immune complexes that integrate pattern recognition, melanization, coagulants, and hemocyte recruitment. *PloS one,* 12**,** e0171447.

Pradel, G. 2007. Proteins of the malaria parasite sexual stages: expression, function and potential for transmission blocking strategies. *Parasitology,* 134**,** 1911-1929.

Price, I., Ermentrout, B., Zamora, R., Wang, B., Azhar, N., Mi, Q., Constantine, G., Faeder, J.R., Luckhart, S. & Vodovotz, Y. 2013. *In vivo*, *in vitro*, and *in silico* studies suggest a conserved immune module that regulates malaria parasite transmission from mammals to mosquitoes. *Journal of theoretical biology,* 334**,** 173-186.

Pukrittayakamee, S., Chotivanich, K., Chantra, A., Clemens, R., Looareesuwan, S. & White, N.J. 2004. Activities of artesunate and primaquine against asexual-and sexual-stage parasites in falciparum malaria. *Antimicrobial agents and chemotherapy,* 48**,** 1329-1334.

Pukrittayakamee, S., Imwong, M., Singhasivanon, P., Stepniewska, K., Day, N.J. & White, N.J. 2008. Effects of different antimalarial drugs on gametocyte carriage in *P. vivax* malaria. *The American journal of tropical medicine and hygiene,* 79**,** 378-384.

Rafinejad, J., Vatandoost, H., Nikpoor, F., Abai, M., Shaeghi, M., Duchen, S. & Rafi, F. 2008. Effect of washing on the bio-efficacy of insecticide-treated nets (ITNs) and long-lasting insecticidal nets (LLINs) against main malaria vector *Anopheles stephens*i by three bioassay methods. *Journal of vector borne diseases,* 45**,** 143.

Raikhel, A.S., Kokoza, V.A., Zhu, J., Martin, D., Wang, S.-F., Li, C., Sun, G., Ahmed, A., Dittmer, N. & Attardo, G. 2002. Molecular biology of mosquito vitellogenesis: from basic studies to genetic engineering of antipathogen immunity. *Insect biochemistry and molecular biology,* 32**,** 1275-1286.

Raikhel, A.S., Miura, K. & Segraves, W.A. 1999. Nuclear receptors in mosquito vitellogenesis. *American zoologist,* 39**,** 722-735.

Ramirez, J.L. & Dimopoulos, G. 2010. The Toll immune signaling pathway control conserved anti-dengue defenses across diverse *Ae. aegypti* strains and against multiple dengue virus serotypes. *Developmental & Comparative Immunology,* 34**,** 625-629.

Ramirez, J.L., Garver, L.S., Brayner, F.A., Alves, L.C., Rodrigues, J., Molina-Cruz, A. & Barillas-Mury, C. 2014a. The role of hemocytes in *Anopheles gambia*e antiplasmodial immunity. *Journal of innate immunity,* 6**,** 119-128.

Ramirez, J.L., Garver, L.S. & Dimopoulos, G. 2009. Challenges and approaches for mosquito targeted malaria control. *Current molecular medicine,* 9**,** 116-130.

Ramirez, J.L., Short, S.M., Bahia, A.C., Saraiva, R.G., Dong, Y., Kang, S., Tripathi, A., Mlambo, G. & Dimopoulos, G. 2014b. Chromobacterium Csp_P reduces malaria and dengue infection in vector mosquitoes and has entomopathogenic and in vitro anti-pathogen activities. *PLoS pathogens,* 10**,** e1004398.

Ramirez, J.L., Souza-Neto, J., Cosme, R.T., Rovira, J., Ortiz, A., Pascale, J.M. & Dimopoulos, G. 2012. Reciprocal tripartite interactions between the *Aedes aegypti* midgut microbiota, innate immune system and dengue virus influences vector competence. *PLoS neglected tropical diseases,* 6**,** e1561.

Ramiro, R.S., Alpedrinha, J., Carter, L., Gardner, A. & Reece, S.E. 2011. Sex and death: the effects of innate immune factors on the sexual reproduction of malaria parasites. *PLoS pathogens,* 7**,** e1001309.

Raz, A., Djadid, N.D. & Zakeri, S. 2013. Molecular characterization of the carboxypeptidase B1 of *Anopheles stephensi* and its evaluation as a target for transmission-blocking vaccines. *Infection and immunity,* 81**,** 2206-2216.

Reese, M.G. 2001. Application of a time-delay neural network to promoter annotation in the *Drosophila melanogaster* genome. *Computers & chemistry,* 26**,** 51-56.

Richards, G. 1981. Insect hormones in development. *Biological Reviews,* 56**,** 501-549.

Richman, A., Bulet, P., Hetru, C., Barillas-Mury, C., Hoffmann, J. & Kafatos, F. 1996. Inducible immune factors of the vector mosquito *Anopheles gambiae*: biochemical purification of a defensin antibacterial peptide and molecular cloning of preprodefensin cDNA. *Insect molecular biology,* 5**,** 203-210.

Rodrigues, J., Brayner, F.A., Alves, L.C., Dixit, R. & Barillas-Mury, C. 2010. Hemocyte differentiation mediates innate immune memory in *Anopheles gambiae* mosquitoes. *Science,* 329**,** 1353-1355.

Rokyta, D.R., Margres, M.J. & Calvin, K. 2015. Post-transcriptional mechanisms contribute little to phenotypic variation in snake venoms. *G3: Genes, Genomes, Genetics,* 5**,** 2375-2382.

Rouhier, N. & Jacquot, J.P. 2005. The plant multigenic family of thiol peroxidases. *Free Radic Biol Med,* 38**,** 1413-21.

Royet, J., Gupta, D. & Dziarski, R. 2011. Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. *Nature Reviews Immunology,* 11**,** 837.

Ryu, J.-H., Kim, S.-H., Lee, H.-Y., Bai, J.Y., Nam, Y.-D., Bae, J.-W., Lee, D.G., Shin, S.C., Ha, E.-M. & Lee, W.-J. 2008. Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in *Drosophi*la. *science,* 319**,** 777-782.

Salazar, C.E., Mills-Hamm, D., Kumar, V. & Collins, F.H. 1993. Sequence of a cDNA from the mosquito *Anopheles gambiae* encoding a homologue of human ribosomal protein S7. *Nucleic acids research,* 21**,** 4147.

Satyavathi, V.V., Minz, A. & Nagaraju, J. 2014. Nodulation: an unexplored cellular defense mechanism in insects. *Cellular signalling,* 26**,** 1753-1763.

Saul, A. 2007. Mosquito stage, transmission blocking vaccines for malaria. *Current opinion in infectious diseases,* 20**,** 476-481.

Schultz, J., Milpetz, F., Bork, P. & Ponting, C.P. 1998. SMART, a simple modular architecture research tool: identification of signaling domains. *Proceedings of the National Academy of Sciences,* 95**,** 5857-5864.

Shanthi, S., Manju, S., Rajakumaran, P. & Vaseeharan, B. 2014a. Molecular cloning of Peroxinectin gene and its expression in response to peptidoglycan and *Vibrio harveyi* in Indian white shrimp *Fenneropenaeus indicus*. *Cell communication & adhesion,* 21**,** 281-289.

Sharma, V. 1999. Current scenario of malaria in India. *Parassitologia,* 41**,** 349-353.

Sharp, P.M. & Li, W.-H. 1986a. An evolutionary perspective on synonymous codon usage in unicellular organisms. *Journal of molecular evolution,* 24**,** 28-38.

Shaw, W.R., Teodori, E., Mitchell, S.N., Baldini, F., Gabrieli, P., Rogers, D.W. & Catteruccia, F. 2014. Mating activates the heme peroxidase HPX15 in the sperm storage organ to ensure fertility in *Anopheles gambia*e. *Proceedings of the National Academy of Sciences,* 111**,** 5854-5859.

Shi, G.Q., Yu, Q.Y. & Zhang, Z. 2012b. Annotation and evolution of the antioxidant genes in the silkworm, *Bombyx mori*. *Archives of insect biochemistry and physiology,* 79**,** 87-103.

Sicard, M., Brugirard-Ricaud, K., Pagès, S., Lanois, A., Boemare, N.E., Brehélin, M. & Givaudan, A. 2004. Stages of infection during the tripartite interaction between *Xenorhabdus nematophila*, its nematode vector, and insect hosts. *Applied and environmental microbiology,* 70**,** 6473-6480.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., Mcwilliam, H., Remmert, M. & Söding, J. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology,* 7**,** 539.

Simon, N., Lasonder, E., Scheuermayer, M., Kuehn, A., Tews, S., Fischer, R., Zipfel, P.F., Skerka, C. & Pradel, G. 2013. Malaria parasites co-opt human factor H to prevent complement-mediated lysis in the mosquito midgut. *Cell host & microbe,* 13**,** 29-41.

Sinden, R. 2002. Molecular interactions between *Plasmodium* and its insect vectors. *Cellular microbiology,* 4**,** 713-724.

Sinden, R.E. & Billingsley, P.F. 2001. *Plasmodium* invasion of mosquito cells: hawk or dove? *Trends in parasitology,* 17**,** 209-211.

Singh, P., Lingala, M.a.L., Sarkar, S. & Dhiman, R.C. 2017. Mapping of Malaria Vectors at District Level in India: Changing Scenario and Identified Gaps. *Vector-Borne and Zoonotic Diseases,* 17**,** 91-98.

Sinka, M.E., Bangs, M.J., Manguin, S., Rubio-Palis, Y., Chareonviriyaphap, T., Coetzee, M., Mbogo, C.M., Hemingway, J., Patil, A.P. & Temperley, W.H. 2012. A global map of dominant malaria vectors. *Parasites & vectors,* 5**,** 69.

Sivakamavalli, J., Selvaraj, C., Singh, S.K. & Vaseeharan, B. 2016a. *In vitro* and *in silico* studies on cell adhesion protein peroxinectin from *Fenneropenaeus indicu*s and screening of heme blockers against activity. *Journal of Molecular Recognition,* 29**,** 186-198.

Smith, R.C., King, J.G., Tao, D., Zeleznik, O.A., Brando, C., Thallinger, G.G. & Dinglasan, R.R. 2016. Molecular profiling of phagocytic immune cells in *Anopheles gambiae* reveals integral roles for hemocytes in mosquito innate immunity. *Molecular & Cellular Proteomics,* 15**,** 3373-3387.

Smith, R.C., Vega-Rodríguez, J. & Jacobs-Lorena, M. 2014. The *Plasmodium* bottleneck: malaria parasite losses in the mosquito vector. *Memórias do Instituto Oswaldo Cruz,* 109**,** 644-661.

Smulevich, G., Jakopitsch, C., Droghetti, E. & Obinger, C. 2006. Probing the structure and bifunctionality of catalase-peroxidase (KatG). *Journal of inorganic biochemistry,* 100**,** 568-585.

Soares, M.P., Silva-Torres, F.A., Elias-Neto, M., Nunes, F.M., Simoes, Z.L. & Bitondi, M.M. 2011. Ecdysteroid-dependent expression of the tweedle and peroxidase genes during adult cuticle formation in the honey bee, Apis mellifera. *PloS one,* 6**,** e20513.

Söderhäll, K. 1982. Prophenoloxidase activating system and melanization-a recognition mechanism of arthropods? A review. *Developmental and comparative immunology,* 6**,** 601-611.

Söderhäll, K., Cerenius, L. & Johansson, M.W. 1994. The prophenoloxidase activating system and its role in invertebrate defence. *Annals of the New York Academy of Sciences,* 712**,** 155-161.

Sologub, L., Kuehn, A., Kern, S., Przyborski, J., Schillig, R. & Pradel, G. 2011. Malaria proteases mediate inside-out egress of gametocytes from red blood cells following parasite transmission to the mosquito. *Cellular microbiology,* 13**,** 897-912.

Song, X., Wang, H., Chen, H., Sun, M., Liang, Z., Wang, L. & Song, L. 2016. Conserved hemopoietic transcription factor Cg-SCL delineates hematopoiesis of Pacific oyster *Crassostrea gigas*. *Fish Shellfish Immunol,* 51**,** 180-8.

Sritunyalucksana, K., Wongsuebsantati, K., Johansson, M. & Söderhäll, K. 2001a. Peroxinectin, a cell adhesive protein associated with the proPO system from the black tiger shrimp, *Penaeus monodon*. *Developmental & Comparative Immunology,* 25**,** 353-363.

Steiner, H., Hultmark, D., Engström, Å., Bennich, H. & Boman, H.G. 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature,* 292**,** 246.

Stern, A., Doron-Faigenboim, A., Erez, E., Martz, E., Bacharach, E. & Pupko, T. 2007. Selecton 2007: advanced models for detecting positive and purifying selection using a Bayesian inference approach. *Nucleic acids research,* 35**,** W506-W511.

Stoletzki, N. 2008. Conflicting selection pressures on synonymous codon use in yeast suggest selection on mRNA secondary structures. *BMC Evolutionary Biology,* 8**,** 224.

Stoletzki, N. & Eyre-Walker, A. 2006. Synonymous codon usage in *Escherichia coli*: selection for translational accuracy. *Molecular biology and evolution,* 24**,** 374-381.

Strand, M. 2008a. Insect hemocytes and their role in immunity. *Insect Immunology Elsevier Inc.*

Strand, M.R. 2008b. The insect cellular immune response. *Insect science,* 15**,** 1-14.

Strand, M.R. & Pech, L.L. 1995. Immunological basis for compatibility in parasitoid-host relationships. *Annu Rev Entomol,* 40**,** 31-56.

Subbarao, S.K. 1998. *Anopheline species complexes in south-east Asia*, WHO Regional Offices for South-East Asia New Delhi.

Suzuki, Y. & Gojobori, T. 1999. A method for detecting positive selection at single amino acid sites. *Molecular biology and evolution,* 16**,** 1315-1328.

Swami, K.K. & Srivastava, M. 2012. Blood meal preference of some anopheline mosquitoes in command and non-command areas of Rajasthan, India. *Journal of arthropod-borne diseases,* 6**,** 98.

Ta, T.H., Hisam, S., Lanza, M., Jiram, A.I., Ismail, N. & Rubio, J.M. 2014. First case of a naturally acquired human infection with *Plasmodium cynomolgi*. *Malaria journal,* 13**,** 68.

Tachida, H. & Kuboyama, T. 1998. Evolution of multigene families by gene duplication: a haploid model. *Genetics,* 149**,** 2147-2158.

Takezaki, N., Figueroa, F., Zaleska-Rutczynska, Z. & Klein, J. 2003. Molecular phylogeny of early vertebrates: monophyly of the agnathans as revealed by sequences of 35 genes. *Molecular Biology and Evolution,* 20**,** 287-292.

Telang, A., Frame, L. & Brown, M.R. 2007. Larval feeding duration affects ecdysteroid levels and nutritional reserves regulating pupal commitment in the yellow fever mosquito *Aedes aegypti* (Diptera: Culicidae). *Journal of Experimental Biology,* 210**,** 854-864.

Theopold, U., Schmidt, O., Söderhäll, K. & Dushay, M.S. 2004. Coagulation in arthropods: defence, wound closure and healing. *Trends in immunology,* 25**,** 289-294.

Thörnqvist, P.-O., Johansson, M.W. & Söderhäll, K. 1994. Opsonic activity of cell adhesion proteins and β-1, 3-glucan binding proteins from two crustaceans. *Developmental & Comparative Immunology,* 18**,** 3-12.

Torres, E. & Ayala, M. 2010. *Biocatalysis based on heme peroxidases: peroxidases as potential industrial biocatalysts*, Springer Science & Business Media.

Tuller, T., Carmi, A., Vestsigian, K., Navon, S., Dorfan, Y., Zaborske, J., Pan, T., Dahan, O., Furman, I. & Pilpel, Y. 2010a. An evolutionarily conserved mechanism for controlling the efficiency of protein translation. *Cell,* 141**,** 344-54.

Tuller, T., Waldman, Y.Y., Kupiec, M. & Ruppin, E. 2010b. Translation efficiency is determined by both codon bias and folding energy. *Proc Natl Acad Sci U S A,* 107**,** 3645-50.

Tyagi, A., Ryme, J., Brodin, D., Farrants, A.K.Ö. & Visa, N. 2009. SWI/SNF associates with nascent pre-mRNPs and regulates alternative pre-mRNA processing. *PLoS genetics,* 5**,** e1000470.

Tzou, P., De Gregorio, E. & Lemaitre, B. 2002. How *Drosophila* combats microbial infection: a model to study innate immunity and host–pathogen interactions. *Current opinion in microbiology,* 5**,** 102-110.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M. & Rozen, S.G. 2012. Primer3—new capabilities and interfaces. *Nucleic acids research,* 40**,** e115-e115.

Valanne, S., Wang, J.-H. & Rämet, M. 2011. The *Drosophila* toll signaling pathway. *The Journal of Immunology,* 186**,** 649-656.

Vanderberg, J.P. & Frevert, U. 2004. Intravital microscopy demonstrating antibody-mediated immobilisation of *Plasmodium berghei* sporozoites injected into skin by mosquitoes. *International journal for parasitology,* 34**,** 991-996.

Vazquez, M., Rodriguez, R. & Zurita, M. 2002. A new peroxinectin-like gene preferentially expressed during oogenesis and early embryogenesis in *Drosophila melanogaster*. *Dev Genes Evol,* 212**,** 526-9.

Venkatrao, V., Kumar, S.K., Sridevi, P., Muley, V.Y. & Chaitanya, R. 2017. Cloning, characterization and transmission blocking potential of midgut carboxypeptidase A in *Anopheles stephensi*. *Acta tropica,* 168**,** 21-28.

Vijay, S., Rawat, M., Adak, T., Dixit, R., Nanda, N., Srivastava, H., Sharma, J.K., Prasad, G.B. & Sharma, A. 2011. Parasite killing in malaria non-vector mosquito *Anopheles culicifacies* species B: implication of nitric oxide synthase upregulation. *PLoS One,* 6**,** e18400.

Vinogradov, A.E. 2003. DNA helix: the importance of being GC-rich. *Nucleic acids research,* 31**,** 1838-1844.

Vizioli, J., Bulet, P., Charlet, M., Lowenberger, C., Blass, C., Müller, H.M., Dimopoulos, G., Hoffmann, J., Kafatos, F. & Richman, A. 2000. Cloning and analysis of a cecropin gene from the malaria vector mosquito, *Anopheles gambiae*. *Insect molecular biology,* 9**,** 75-84.

Vizioli, J., Bulet, P., Hoffmann, J.A., Kafatos, F.C., Müller, H.-M. & Dimopoulos, G. 2001a. Gambicin: a novel immune responsive antimicrobial peptide from the malaria vector *Anopheles gambiae*. *Proceedings of the National Academy of Sciences,* 98**,** 12630-12635.

Vizioli, J., Richman, A.M., Uttenweiler-Joseph, S., Blass, C. & Bulet, P. 2001b. The defensin peptide of the malaria vector mosquito *Anopheles gambiae*: antimicrobial activities and expression in adult mosquitoes. *Insect biochemistry and molecular biology,* 31**,** 241-248.

Vizzini, A., Parrinello, D., Sanfratello, M.A., Mangano, V., Parrinello, N. & Cammarata, M. 2013. *Ciona intestinalis* peroxinectin is a novel component of the peroxidase-cyclooxygenase gene superfamily upregulated by LPS. *Dev Comp Immunol,* 41**,** 59-67.

Vlachou, D., Schlegelmilch, T., Christophides, G.K. & Kafatos, F.C. 2005. Functional genomic analysis of midgut epithelial responses in *Anopheles* during *Plasmodium* invasion. *Current Biology,* 15**,** 1185-1195.

Volanakis, J.E. 1998. *The human complement system in health and disease*, Informa Health Care.

Volohonsky, G., Hopp, A.-K., Saenger, M., Soichot, J., Scholze, H., Boch, J., Blandin, S.A. & Marois, E. 2017. Transgenic Expression of the Anti-parasitic Factor TEP1 in the Malaria Mosquito *Anopheles gambiae*. *PLoS pathogens,* 13**,** e1006113.

Volz, J., Osta, M.A., Kafatos, F.C. & Müller, H.-M. 2005. The roles of two clip domain serine proteases in innate immune responses of the malaria vector *Anopheles gambiae*. *Journal of Biological Chemistry,* 280**,** 40161-40168.

Wang, C.S., Ashton, N.N., Weiss, R.B. & Stewart, R.J. 2014. Peroxinectin catalyzed dityrosine crosslinking in the adhesive underwater silk of a casemaker caddisfly larvae, *Hysperophylax occidentalis*. *Insect Biochem Mol Biol,* 54**,** 69-79.

Wang, S., Dos-Santos, A.L., Huang, W., Liu, K.C., Oshaghi, M.A., Wei, G., Agre, P. & Jacobs-Lorena, M. 2017. Driving mosquito refractoriness to *Plasmodium falciparum* with engineered symbiotic bacteria. *Science,* 357**,** 1399-1402.

Wang, Y., Gilbreath Iii, T.M., Kukutla, P., Yan, G. & Xu, J. 2011. Dynamic gut microbiome across life history of the malaria mosquito *Anopheles gambiae* in Kenya. *PloS one,* 6**,** e24767.

Warnecke, T. & Hurst, L.D. 2007. Evidence for a trade-off between translational efficiency and splicing regulation in determining synonymous codon usage in *Drosophila melanogaster*. *Molecular biology and evolution,* 24**,** 2755-2762.

Waterhouse, R.M., Kriventseva, E.V., Meister, S., Xi, Z., Alvarez, K.S., Bartholomay, L.C., Barillas-Mury, C., Bian, G., Blandin, S. & Christensen, B.M. 2007. Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. *science,* 316**,** 1738-1743.

Wekesa, J.W., Copeland, R.S. & Mwangi, R.W. 1992. Effect of *Plasmodium falciparum* on blood feeding behavior of naturally infected *Anopheles* mosquitoes in western Kenya. *The American journal of tropical medicine and hygiene,* 47**,** 484-488.

White, B.J., Collins, F.H. & Besansky, N.J. 2011. Evolution of *Anopheles gambiae* in relation to humans and malaria. *Annual review of ecology, evolution, and systematics,* 42**,** 111-132.

White, G. 1974. *Anopheles gambiae* complex and disease transmission in Africa. *Transactions of the Royal Society of Tropical Medicine and hygiene,* 68**,** 278-298.

White, N. 2008. *Plasmodium knowlesi*: the fifth human malaria parasite. The University of Chicago Press.

Whitten, M.M., Tew, I.F., Lee, B.L. & Ratcliffe, N.A. 2004. A novel role for an insect apolipoprotein (apolipophorin III) in β-1, 3-glucan pattern recognition and cellular encapsulation reactions. *The Journal of Immunology,* 172**,** 2177-2185.

William, T., Rahman, H.A., Jelip, J., Ibrahim, M.Y., Menon, J., Grigg, M.J., Yeo, T.W., Anstey, N.M. & Barber, B.E. 2013. Increasing incidence of *Plasmodium knowlesi* malaria

following control of *P. falciparum* and *P. vivax* malaria in Sabah, Malaysia. *PLoS neglected tropical diseases,* 7**,** e2026.

Willyard, C. 2018. Malaria eradication: blocking transmission to mosquitoes. *Stroke,* 13**,** 57.

Windbichler, N., Menichelli, M., Papathanos, P.A., Thyme, S.B., Li, H., Ulge, U.Y., Hovde, B.T., Baker, D., Monnat, R.J. & Burt, A. 2011. A synthetic homing endonuclease-based gene drive system in the human malaria mosquito. *Nature,* 473**,** 212.

Yamamura, T., Takahashi, S., Satoh, T., Iwabuchi, K. & Okazaki, T. 2011. Regulatory mechanism of silkworm hemocyte adhesion to organs. *Zoolog Sci,* 28**,** 420-9.

Yamauchi, L.M., Coppi, A., Snounou, G. & Sinnis, P. 2007. Plasmodium sporozoites trickle out of the injection site. *Cellular microbiology,* 9**,** 1215-1222.

Yang, C.-C., Lu, C.-L., Chen, S., Liao, W.-L. & Chen, S.-N. 2015. Immune gene expression for diverse haemocytes derived from pacific white shrimp, *Litopenaeus vannamei*. *Fish & shellfish immunology,* 44**,** 265-271.

Yue, F., Zhou, Z., Wang, L., Wang, M. & Song, L. 2014. A conserved zinc finger transcription factor GATA involving in the hemocyte production of scallop *Chlamys farreri*. *Fish & shellfish immunology,* 39**,** 125-135.

Zamocky, M., Gasselhuber, B., Furtmuller, P.G. & Obinger, C. 2014. Turning points in the evolution of peroxidase-catalase superfamily: molecular phylogeny of hybrid heme peroxidases. *Cell Mol Life Sci,* 71**,** 4681-96.

Zámocký, M., Hofbauer, S., Schaffner, I., Gasselhuber, B., Nicolussi, A., Soudi, M., Pirker, K.F., Furtmüller, P.G. & Obinger, C. 2015. Independent evolution of four heme peroxidase superfamilies. *Archives of biochemistry and biophysics,* 574**,** 108-119.

Zamocky, M., Jakopitsch, C., Furtmüller, P.G., Dunand, C. & Obinger, C. 2008. The peroxidase–cyclooxygenase superfamily: reconstructed evolution of critical enzymes of the innate immune system. *Proteins: Structure, Function, and Bioinformatics,* 72**,** 589-605.

Zámocký, M. & Obinger, C. 2010. Molecular phylogeny of heme peroxidases. *Biocatalysis based on heme peroxidases.* Springer.

Zdybicka-Barabas, A., Sowa-Jasiłek, A., Stączek, S., Jakubowicz, T. & Cytryńska, M. 2015. Different forms of apolipophorin III in *Galleria mellonella* larvae challenged with bacteria and fungi. *Peptides,* 68**,** 105-112.

Zhang, G., Wang, H., Shi, J., Wang, X., Zheng, H., Wong, G.K., Clark, T., Wang, W., Wang, J. & Kang, L. 2007. Identification and characterization of insect-specific proteins by genome data analysis. *BMC Genomics,* 8**,** 93.

Zhang, J.-Y., Liu, Q.-H. & Huang, J. 2014. Multiple proteins of White spot syndrome virus involved in recognition of β-integrin. *Journal of biosciences,* 39**,** 381-388.

Zhang, P., Cao, G., Sheng, J., Xue, R. & Gong, C. 2012. BmTGIF, a *Bombyx mori* homolog of *Drosophila* DmTGIF, regulates progression of spermatogenesis. *PloS one,* 7**,** e47861.

Zhang, X., An, C., Sprigg, K. & Michel, K. 2016. CLIPB8 is part of the prophenoloxidase activation system in *Anopheles gambiae* mosquitoes. *Insect biochemistry and molecular biology,* 71**,** 106-115.

Appendix

List of Publications in cited Journals (SCI)

1. **Choudhury TP**, Gupta L & Kumar S. 2019. Identification, characterization and expression analysis of *Anopheles stephensi* double peroxidase. **Acta Tropica**, 190, 210-219

2. Kakani P, Kajla M, **Choudhury TP**, Gupta L and Kumar S, *Anopheles stephensi* Dual Oxidase Silencing Activates the Thioester-Containing Protein 1 Pathway to Suppress *Plasmodium* Development. **Journal of Innate Immunity**, pp.1-10.

3. Kajla M, **Choudhury TP**, Kakani P, Gupta K, Dhawan R, Gupta K and Kumar S. 2016. Silencing of *Anopheles stephensi* Heme Peroxidase HPX15 Activates Diverse Immune Pathways to Regulate the Growth of Midgut Bacteria. **Frontiers in Microbiology.** 7:1351. doi: 10.3389/fmicb.2016.01351.

4. Kajla M[#], Kakani P[#], **Choudhury TP**, Gupta K, Gupta L, Kumar S. 2016. Characterization and expression analysis of gene encoding heme peroxidase HPX15 in major Indian malaria vector *Anopheles stephensi* (Diptera: culicidae). **Acta Tropica**. 158: 107-116.doi:10.1016/j.actatropica.2016.02.028. (# Equal contribution).

5. Kajla M, Kakani P, **Choudhury TP**, Kumar V, Gupta K, Dhawan R, Gupta L and Kumar S. 2017. *Anopheles stephensi* Heme Peroxidase HPX15 Suppresses Midgut Immunity to Support *Plasmodium* Development. **Frontiers in Immunology**. 8:249. doi: 10.3389/fimmu.2017.00249.

6. Dhawan R**,** Gupta K, Kajla M, Kakani P, **Choudhury TP**, Kumar S, Kumar V and Gupta L. 2017. Apolipophorin-III Acts as a Positive Regulator of *Plasmodium* Development in *Anopheles stephensi*. ***Frontiers in Physiology.*** 8:185. doi: 10.3389/fphys.2017.00185

7. Kajla M, Gupta K, Kakani P, Dhawan R, **Choudhury TP**, Gupta L, Gakhar SK, Kumar S. 2015. Identification of an *Anopheles* Lineage-Specific Unique Heme Peroxidase HPX15: A Plausible Candidate for Arresting Malaria Parasite Development. **Phylogenetics & Evolutionary Biology**. 3: 160. doi:10.4172/2329- 9002.1000160.

8. Kajla M, Kakani P, **Choudhury TP**, Gupta K, Dhawan R, Gakhar SK, Gupta L, Kumar S. 2014. Characterization of Anopheline unique peroxidase and its role in regulation of *Plasmodium* development. **Malaria Journal**. 13(suppl 1):P49.

9. Dhawan R, Gupta K, Kajla M, Kumar S, Gakhar SK, Kakani P, **Choudhury TP** and Gupta L. 2015. Molecular characterization of SOCS gene and its expression analysis on *Plasmodium berghei* infection in *Anopheles culicifacies*. **Acta Tropica**. 152:170–175.

Research Articles communicated/in pipeline

1. **Choudhury TP**, Kajla M, Kakani P, Gupta L and Kumar S. 2018. Tracing the phylogeny of a biparttite heme peroxidase in *A. stephensi*.

2. **Choudhury TP**, Kajla M, Kakani P, Gupta L and Kumar S. 2018. Unique role of AsDBLOX in mosquito hemolymph immunity.

List of Publications in International and National conferences

1. **Choudhury TP**, Kajla M, Kakani P, Gupta K, Dhawan R, Gupta L and Kumar S. Exploring the role of *Anopheles stephensi* heme peroxidase in mosquito innate immunity. 6th World Congress on Biotechnology. October 05-07, **2015** New Delhi, India. J Biotechnol Biomater DOI: 10.4172/2155-952X.C1.044.

2. **Choudhury TP**, Kajla M, Kakani P, Gupta K, Dhawan R, Gupta L and Kumar S. Exploring the role of *Anopheles stephensi* heme peroxidase in mosquito innate immunity. International Vaccine Conference, Nov 27-29, **2017**, ICGEB, New Delhi, India.

3. **Choudhury TP**, Kajla M, Kakani P, Gupta K, Dhawan R, Gupta L and Kumar S. Exploring the role of *Anopheles stephensi* heme peroxidase in mosquito innate immunity. BITS Conference on Gene and Genome Regulation (BCGGR 2016). February 18-20, **2016** BITS Pilani, Pilani Campus, India.

4. **Choudhury TP,** Kajla M**,** Gupta K, Dhawan R, Kakani P**,** Gupta L and Kumar S. Exploring the role of *Anopheles stephensi* Heme Peroxidase in Mosquito Innate Immunity. VIIIth National conference of Indian Academy of Tropical Parasitology TROPACON-2014, 20th – 22nd Nov, **2014**, RMRC, Dibrugarh, Assam, India.

5. Kakani P**,** Kajla M, **Choudhury TP**, Gupta K, Dhawan R, Gupta L and Kumar S. 2016. *Anopheles stephensi* dual oxidase maintains microbial homeostasis in blood fed midgut. ***Journal of Bacteriology & Parasitology***. 7:3(Suppl):P107. 2nd International Conference on Parasitology, August 01-03, 2016, Manchester, UK.

6. Kakani P, Yadav A, Kajla M, **Choudhury TP**, Gupta K, Dhawan R, Gupta L and Kumar S. Comparative analysis of two duplicated heme-peroxidase AsHPX10 and AsHPX11 genes in *Anopheles stephensi* having high divergent mRNA expression. BITS Conference on Gene and Genome Regulation (BCGGR 2016). February 18-20, 2016 BITS Pilani, Pilani Campus, India.

7. Kakani P**,** Kajla M**,** Gupta K, Dhawan R, **Choudhury TP**, Gupta L and Kumar S. Characterization of *Anophelines* Peroxidase- Role in Development and Immunity against Blood- borne antigens. VIIIth National conference of Indian Academy of Tropical Parasitology TROPACON-2014, 20th – 22nd Nov, **2014**, RMRC, Dibrugarh, Assam, India.

8. Kajla M, Kakani P, **Choudhury TP**, Gupta K, Dhawan R, Gupta L and Kumar S. HPX15 modulates immune response against gut bacteria of *Anopheles stephensi*. BITS Conference on Gene and Genome Regulation (BCGGR 2016). February 18-20, **2016** BITS Pilani, Pilani Campus, India.

9. Vaswani D**,** Kajla M**,** Kakani P, Dhawan R, Gupta K, **Choudhury TP ,** Gupta L and Kumar S.Characterization of Inhibitory Effects of Herbal Extracts on *Aedes aegypti* Egg Chorion Melanization and Hardening. VIIIth National conference of Indian Academy of Tropical Parasitology TROPACON-2014, 20th – 22nd Nov, **2014**, RMRC, Dibrugarh, Assam, India.

10. Dhawan R, Soam A, Gupta K, Kajla M, Kakani P, **Choudhury TP**, Kumar S, Gupta L.Role of Mosquito Immunity against Parasite Development: An Expedition of Potent Immune Molecules of *Anopheles* Mosquitoes. VIIIth National conference of Indian Academy of Tropical Parasitology TROPACON-2014, 20th – 22nd Nov, **2014**, RMRC, Dibrugarh, Assam, India.

11. Gupta L, Gupta K, Dhawan R, Kumar S, Kakani P, Kajla M, and **Choudhury TP**. *Anopheles* STAT pathway duplication and its role in immunity against *Plasmodium*. VIIIth National conference of Indian Academy of Tropical Parasitology TROPACON-2014, 20th – 22nd Nov, **2014**, RMRC, Dibrugarh, Assam, India.

12. Kumar S, Kakani P, Kajla M, Gupta K, **Choudhury TP**, Dhawan R, and Gupta L. Compartment-specific immune responses in malaria vector determine the vectorial capacity. 25th National Congress of Parasitology on “Global Challenges in the Management of Parasitic Diseases (GCMPD), Central Drug Research Institute (CDRI), OP 27, 16-18th Oct. **2014**, Lucknow, UP, India.

13. Gupta K, Dhawan R, Kajla M, **Choudhury TP**, Kakani P, Kumar S. and Gupta L**.** Genomic diversity of STAT (Signal Transducer and Activator of Transcription) gene in different *Anopheles* mosquito species. 25th National Congress of Parasitology on “Global Challenges in the Management of Parasitic Diseases (GCMPD), Central Drug Research Institute (CDRI), YS 2, 16-18th Oct. **2014**, Lucknow, UP, India

14. Kajla M, Kakani P, **Choudhury TP**, Gupta K, Dhawan R, Gupta L, Gakhar SK, Gupta L, Kumar S. Characterization of *Anopheline* unique peroxidase and its role in regulation of *Plasmodium* development. Challenges in Malaria Research: Core science and innovation. Organized by BioMed Central, The Open Acess Publisher, September 22-24, **2014** at Oxford University, Oxford, UK.

Biography of supervisor Prof. Prabhat Nath Jha

Dr. Jha has been working as Associate Professor at Department of Biological Sciences, BITS Pilani since April 2008. Further, he was awarded Raman Fellowship under India-USA knowledge initiative program in 2013 and worked with Frank Dazzo at Michigan state university for a year where he studied microbial diversity using CMEIAS suites of software, and bacterial community analysis using Next Generation Sequencing platform. Considering research as a soul of teaching, Dr. Jha has been working in the field of plant-microbe interaction, biodegradation of polychlorinated biphenyls, microbial ecology, and molecular mechanism of host-pathogen interaction.

Distinguished Publications of Dr. Prabhat Nath Jha:

1. A Singh RP, Nalwaya S, **Jha PN**. **2017** The draft genome sequence of the plant growth promoting rhizospheric bacterium Enterobacter cloacae SBP-8. **Genome Data** 10.1016/j.gdata.2017.03.006.

2. Singh RP, Jha P, **Jha PN**. **2017**. Analysis of fatty acid composition of PGPR Klebsiella sp. SBP-8 and its role in ameliorating salt stress in wheat. **Symbiosis** 10.1007/s13199-017-0477-4

3. Singh RP, Jha P, **Jha PN**. **2017**. Bio-inoculation of Plant growth-promoting rhizobacterium Enterobacter cloacae ZNP-3 increased resistance against salt and temperature stress in wheat plant (Triticum aestivum L.). **Journal of Plant Growth Regulation**. 10.1007/s00344-017-9683-9

4. Ashok P, Chander S, Chow LMC, Wong ILK, Singh RP, **Jha PN**, Sankaranarayanan M. **2016**. Synthesis and in-vitro anti-leishmanial activity of (4-arylpiperazin-1-yl)(1-(thiophen-2-yl)-9H-pyrido[3,4-b]indol-3-yl)methanone derivatives. **Bioorganic Chemistry**. http://dx.doi.org/10.1016/j.bioorg.2016.11.013

5. Baig N, Singh RP, **Jha PN**, Sah AK. **2016**. Synthesis of Glucose- Derived Glycoconjugates and Studies on Their Antimicrobial Activities: Mechanistic Insight**. Chemistry Select**. 10.1002/slct.201600786

Biography of Co-Supervisor Prof. Sanjeev Kumar

Prof. Sanjeev Kumar is presently working as Professor and Head of Department of Biotechnology and Dean of Pharmaceutical Sciences at Ch. Bansi Lal University (CBLU), Bhiwani, Haryana. He has been an Associate Dean, SRCD, BITS-Pilani and Associate Professor, Department of Biological Sciences, BITS-Pilani, Rajasthan. He received his PhD. degree in 1999 from Banaras Hindu University, Varansi, UP, India. He has been a Research Fellow (a Federal Full time equivalent) at Laboratory of Malaria and Vector Research, National Institutes of Health (NIH), Rockville, MD, USA. He has been also a Post-Doctoral Fellow in in the Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, USA. He has been an INSERM foreign scientist in the lymphoid cell differentiation and gene recombination laboratory, INSERM-CNRS Centre d'Immunologie Marseille-Luminy (CIML), Marseille, France. He has been awarded with various achievements and fellowships. He has been involved in the research and teaching for more than 17 years. His area of interest includes understanding the Cellular biology and biochemistry of mosquito (malaria vector) innate immune responses during *Plasmodium* (malaria parasite) infection and importance of heme peroxidases against *Plasmodium* and bacteria. He has published various research articles in peer reviewed international journals.

The work was supervised by Prof. Sanjeev Kumar in his DST Sponsored project and due to last moment shifting of Supervisor, he was made the Co-Supervisor.

Distinguished Publications of Prof. Sanjeev Kumar:

1. **Kumar S**, Molina-Cruz A, Gupta L, Rodrigues J, Barillas-Mury C. **2010**. A Peroxidase/Dual Oxidase system modulates midgut epithelial immunity in *Anopheles gambiae*. **Science** 327:1644-1648.
2. **Kumar S**, Gupta L, Han YS, Barillas-Mury C. **2004**. Inducible peroxidases mediate nitration of *Anopheles* midgut cells undergoing apoptosis in response to *Plasmodium* invasion. **Journal of Biological Chemistry** 279:53475-53482.
3. **Kumar S,** Christophides GK, Cantera R, Charles B, Han YS, Meister S, Dimopoulos G, Kafatos FC, Barillas-Mury C. **2003**. The role of reactive oxygen species on *Plasmodium* melanotic encapsulation in *Anopheles gambiae*. **PNAS** 100:14139-14144.
4. Spicuglia S, **Kumar S**, Yeh JH, Vachez E, Chasson L, Gorbatch S, Cautres J, Ferrier P. **2002**. Promoter activation by enhancer-dependent and -independent loading of activator and coactivator complexes. **Molecular Cell** 10: 1479-1487.
5. Kajla M, Kakani P, Choudhury TP, Kumar V, Gupta K, Dhawan R, Gupta L and **Kumar S**. **2017**. *Anopheles stephensi* Heme Peroxidase HPX15 Suppresses Midgut Immunity to Support *Plasmodium* Development. **Frontiers in Immunology**. 8:249.
6. Kajla M, Choudhury TP, Kakani P, Gupta K, Dhawan R, Gupta K and **Kumar S**. **2016**. Silencing of *Anopheles stephensi* Heme Peroxidase HPX15 Activates Diverse Immune Pathways to Regulate the Growth of Midgut Bacteria. **Frontiers in Microbiology.** 7:1351

Biography of Tania Pal Choudhury

Tania Pal Choudhury has completed her B. Sc. in Zoology from Bhairab Ganguly College affiliated to Calcutta University, Kolkata in 2009 and M.Sc. Zoology in 2011 from West Bengal State University, West Bengal, India. She worked as JRF/SRF in DST funded project entitled “Molecular characterization of *Anopheles stephensi* gut epithelial peroxidases and their role in modulating immunity against bacterial and fungal antigens” at BITS Pilani, Rajasthan. She received all India rank 665 in GATE 2013. She has cleared national level examination CSIR/UGC LS. She has been enrolled as doctoral fellow at Birla Institute of Technology and Science (BITS), Pilani, since January, 2014. Her area of interest includes understanding the importance of heme peroxidases in innate immune responses against *Plasmodium* and bacteria. She has published various research articles in well renowned international journals.