AMERICAN UNIVERSITY OF BEIRUT

EVALUATING THE CONTRIBUTION OF MELANIZATION TO ANTIBACTERIAL DEFENSE IN THE MALARIA VECTOR *ANOPHELES GAMBIAE*

by
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AN ABSTRACT OF THE THESIS OF

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Title: Evaluating the Contribution of Melanization to Antibacterial Defense in the Malaria Vector *Anopheles gambiae*

Melanization is a potent immune response used by arthropods. It involves the deposition of melanin on the surface of pathogens and is also involved in wound healing. Innate immunity of *Anopheles gambiae* is characterized by recognition of pathogens by pattern recognition receptors (PRRs) that bind to pathogen associated molecular patterns (PAMPs). Thus, this interaction will lead to activating the serine protease cascades that are involved in converting prophenoloxidase (PPO) into active phenoloxidase (PO) that is involved in melanization. The main factors involved in the serine protease cascades belong to clip-domain serine proteases family(CLIPs).

Previous studies have shown that *A. gambiae* rarely melanize *Plasmodium* parasites but they do melanize bacteria. However, the role of melanization in the antibacterial defense is still controversial. A previous study have shown that melanization in *Anopheles gambiae* is not required for resistance and tolerance of mosquitoes to infections with *E. coli* and *S. aureus* bacteria, which are model bacteria and not of mosquito pathogens. Thus the need to further characterize this response in *Anopheles gambiae* against a broader spectrum of species is required. Here, we investigated the role of melanization in antibacterial defense to *Streptococcus iniae*, a Gram-positive fish pathogen, as a model bacterium. Here we show that melanization plays an important role in the defense against *Streptococcus iniae* bacteria. Western blot analysis revealed that CLIPA8 is cleaved in the hemolymph of mosquitoes infected with *Streptococcus iniae*. Moreover, comparing the dynamics of CLIPA8 cleavage between the Gram-negative *E. coli* and the Gram-positive *S. iniae* shows that the later triggers more cleavage of CLIPA8 protein. *S. iniae* triggered also a consistently higher hemolymph PO activity than E. coli, implying more melanization is occurring. Strikingly, *CLIPA8*kd mosquitoes showed significantly more compromised survival following *S. iniae* infection compared to *LazZ* kd, while *E. coli* or *S. aureus* infected mosquitoes showed a similar survival pattern to *LacZ*kd controls. Interestingly, *CLIPA8* kd mosquitoes contained less *S. iniae* DNA compared to LacZ kd controls and that at several days post-infection. This indicates that *CLIPA8* kd mosquitoes are more resistant but less tolerant to S. iniae infections than controls.

The results obtained suggest that melanization affects bacterial tolerance and resistance in a species-dependent way. Therefore, to obtain a deeper in sight into the contribution of melanization to antibacterial defense it is important to use a broad panel of bacterial species.

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ABBREVIATIONS

A. albimanus	*Anopheles albimanus*
A. hydrophila	*Anopheles hydrophila*
A. stephensi	*Anopheles stephensi*
Ae. aegypti	*Aedes aegypti*
An or A. gambiae	*Anopheles gambiae*
AMP	Antimicrobial peptide
APL1C	Anopheles Plasmodium-responsive leucine-rich repeat protein 1C
BHI	Brain Heart infusion
CEC	Cecropin
cfu	Colony forming unit
CLIP	Clip-domain serine protease
CTAB	Cetyl trimethyl ammonium bromide
CTL	C-type lectin
D. melanogaster	*Drosophila melanogaster*
DEF	Defensin
DIF	Dorsal-related immunity factor
dopa	3,4-dihydroxyphenylalanine
DNA	Deoxyribonucleic acid
DPT	Diptericin
Dscam	Down syndrome cell adhesion molecule
dsRNA	Double-stranded RNA
DTT	Dichlorodiphenyltrichloroethane

FREP or FBN	Fibrinogen-related protein
E. coli	*Escherichia coli*
GAM	Gambicin
GFP	Green fluorescence protein
GNBP	Gram-negative binding protein
HEG	Homing endonuclease gene
HPX2	Heme peroxidase 2
Ig	Immunoglobulin
Imd	Immune deficiency
JNK	Jun-N-terminal kinase
kDa	Kilo Dalton
kd	Knockdown
LB	Luria Bertani
lctO	Lactate oxidase
LPS	Lipopolysaccharide
LRIM1	Leucine-rich repeat immune protein 1
LRR	Leucine-rich repeat
M. sexta	*Manduca sexta*
NADPH	Nicotinamide adenine dinucleotide phosphate
NOS	Nitric oxide synthase
NOX5	NADPH oxidase 5
OD	Optical density
P. berghei	*Plasmodium berghei*
P. falciparum	*Plasmodium falciparum*

P. knowlesi	*Plasmodium knowlesi*
P. malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
P. vivax	*Plasmodium vivax*
PAMP	Pathogen-associated molecular pattern
PAP	Prophenoloxidase activating protease
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
PO	Phenoloxidase
PPAE	Prophenoloxidase-activating enzyme
PPAF	Prophenoloxidase-activating factor
PPO	Prophenoloxidase
PRR	Pattern recognition receptor
RBC	Red blood cell
RNA	Ribonucleic acid
RNAi	Ribonucleic acid-interference
S. aureus	*Staphylococcus aureus*
S. iniae	*Streptococcus iniae*
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPH	Serine protease homolog
SRPN	Serine protease inhibitor or serpin
STAT	Signal transducers and activators of transcription

TE	Transposable element
TEP	Thioester-containing protein
WHO	World Health Organization

CHAPTER I

INTRODUCTION

Malaria, according to the WHO, is one of the three major infectious diseases that has a high rate of morbidity and mortality especially in children below the age of 5 years in sub-Saharan Africa. Malaria is caused by a protozoan parasite of the genus *Plasmodium* that is transmitted to humans by more than 30 anopheline species. Five species of *Plasmodium* can cause disease in humans. Most cases are caused by *P. falciparum* or *P. vivax*, while infections with *P. ovale*, *P. malariae* and *P. knowlesi* are less prevalent (Kantele and Jokiranta, 2011). In 2010, the occurrence of malaria cases was estimated to be around 219 million cases with an uncertainty range of 154-289 million and around 660,000 deaths caused by malaria with an uncertainty range of 610,000 -971,000 deaths. In 2011, 3.3 billion people were at risk of getting malaria (WHO, 2010).

A. Malaria Life Cycle

The *Plasmodium* life cycle is complex requiring a vertebrate and an invertebrate (mosquito) host to complete its cycle. It is composed of two stages: the asexual one occurs in the vertebrate host while sexual stage takes place in the mosquito vector.

Briefly, in the asexual stage, an infected mosquito bites a vertebrate host inoculating sporozoites into the dermis from where they gain quick access to the blood circulation reaching into the liver. In the liver, the pre-erythrocytic phase takes place whereby the sporozoites differentiate and proliferate to produce exoerythrocytic

schizonts containing each multiple merozoites with lemon-like morphology that are capable of infecting red blood cells (RBCs). Following the rupture of hepatocytes, merozoites are released into the bloodstream where they infect RBCs and initiate the erythrocytic cycle. In this cycle, a merozoite infecting an RBC first develops into a ring which matures to form a trophozoite that eventually undergoes several mitotic divisions forming a schizont. The schizont ruptures and releases merozoites that infect new red blood cells. Some ring stage parasites develop into sexual stage gametocytes that are infectious to the mosquito vector.

In the sexual stage of the cycle, the parasite goes through several developmental changes in the mosquito vector before it is successfully transmitted to the vertebrate host. It starts with the ingestion of infected blood by a naive mosquito from an infected vertebrate host. At the level of the midgut, male and female gametocytes quickly differentiate into gametes. The chemical environment of the midgut plays an important role in this differentiation, which is followed by the formation of a zygote (Billker et al., 1998; Billker et al., 1997). Later, the zygote transforms into a motile ookinete which crosses the midgut of the mosquito through the peritrophic membrane until it reaches the basal lamina where it arrests and develops into an oocyst. Two meiotic divisions take place, the first in the zygote and the second in the ookinete resulting in four haploid nuclei in the latter. In each oocyst, multiple rounds of mitotic divisions result in the production of thousands of sporozoites. Subsequently, the oocyst ruptures after around two weeks post-infection and release the sporozoites into the haemoceol. The sporozoites migrate to the salivary gland lumen where they further mature rendering the mosquito infectious to a new vertebrate host. The entire cycle in the mosquito takes between 17 and 21 days. It is worth noting that the parasite journey

inside the vector is not passive since the vector mounts a potent immune response resulting in the elimination of the majority of invading ookinetes.

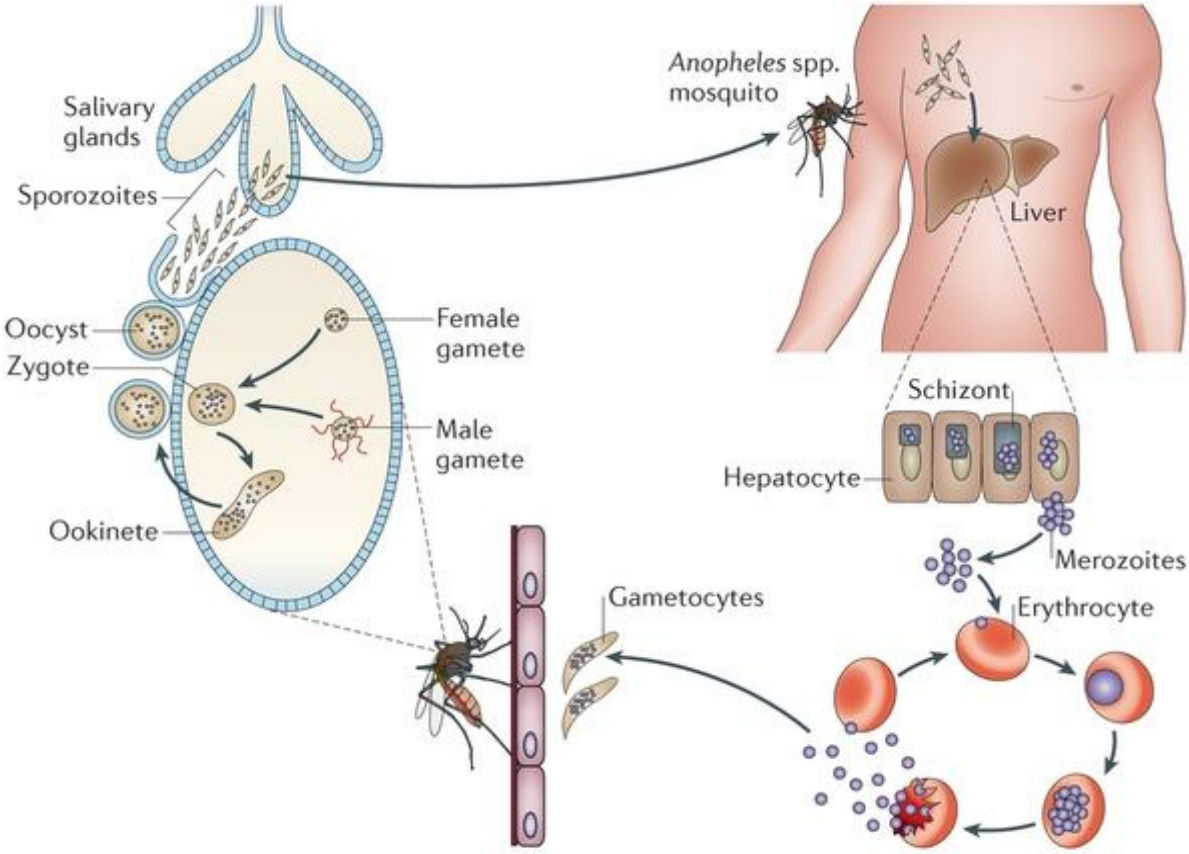

Figure 1. Plasmodium life cycle in its human host and mosquito vector. Figure adapted from (Flannery et al., 2013)

B. Approaches Used to Fight Malaria

Strategies to fight malaria have been traditionally targeted towards vector control through indoor residual spraying, or use of insecticide-impregnated nets and chemotherapy with antimalarial drugs. However, both of these strategies were hindered due to insecticide resistance in the vector and drug resistance in the parasite (Fidock et al., 2008; Koekemoer et al., 2011). During the 1950s and 1960s, dichlorodiphenyltrichloroethane (DDT) was used at a large scale as an insecticide and helped eradicate malaria from several temperate regions around the globe. The use of

DDT was later restricted due to the environmental and health hazards, and currently malaria vector control is almost entirely dependent on a single class of insecticides, the pyrethroids (Ranson et al., 2011). Pyrethroids are the only insecticides approved for use on insecticide-treated bednets, However due to their widespread use against agricultural pests worldwide there has been increasing reports of resistance in malaria vectors against these insecticides during the last decade (Santolamazza et al., 2008). Most currently available malaria drugs target the asexual blood stages of the parasite's life cycle, however, as with insecticides, resistance has been observed to different extents for every existing approved antimalarial compound (Neafsey, 2013). Among the main drugs used nowadays are chloroquine, mefloquine (Lariam), atovaquone and proguanil (Malarone) and Sulfadoxine and pyrimethamine (Fansidar). In addition to resistance, another important obstacle in front of malaria eradication is that both *P. vivax* and *P. ovale* form hypnozoites (dormant liver stage) which are more difficult to treat and the only drug efficient against hypnozoites is primaquine which exhibits several side effects in some patients (Wells et al., 2010). Hence, new and safer drugs are needed that can target all stages of the *Plasmodium* life cycle if the goal is to totally eradicate the disease in the future (Kappe et al., 2010; Tanner and de Savigny, 2008).

Vaccine design against malaria have been also hampered by the complex biology of the malaria parasite. The most advanced vaccine which showed potential signs of success is RTS,S/AS01 which directed against *P. falciparum* . RTS,S is currently in phase 3 trial and full results from this trial are expected in 2015 (Moorthy et al., 2013). The pilot trials concluded that this vaccine provided around 50% protection against clinical and severe malaria episodes in children vaccinated at the age of 5 to 17 months. When the age of vaccinated children was between 6 and 12 weeks, the efficiency was around 30% (Agnandji et al., 2011; Moorthy et al., 2013). Although this

percentage is considered low, it can be the first step towards limiting the spread of malaria and preventing death among children. Some major obstacles facing vaccine development are the polymorphic nature of the parasites' antigens and poor immunogenicity of vaccine candidates (Good, 2009).

In front of this complex situation, more original control strategies are being investigated especially regarding vector control. This was facilitated by the advancement of genetic tools to manipulate malaria vectors and sequencing of the full genome of the major vector in Africa, *Anopheles gambiae*, which allowed better understanding of the biology of malaria vectors and their interactions with malaria parasites. One of the approaches investigated in this context is the production of transgenic mosquitoes that can either abolish the transmission of malaria or suppress the population of mosquitoes that can transmit the parasite. Creating transgenic mosquitoes relies on introducing a gene into the insect genome so that the development of the pathogen will be hindered. The first stable transformation of mosquitoes was performed in the dengue vector *Aedes aegypti* (Coates et al., 1998; Jasinskiene et al., 1998) and was soon followed by successful transformation of the malaria vectors *A. stephensi* (Catteruccia et al., 2000), *A. gambiae* (Grossman et al., 2001) and *A. albimanus* (Perera et al., 2002).

Transgenic mosquitoes can be constructed to carry effector genes from mosquitoes exhibiting enhanced immunity, from other organisms, or from single-chain antibodies. For example two key proteins in the mosquito innate immune system, TEP1 and LRIM1, have been shown to play a role in ookinete lysis and melanization where silencing either gene led to increase in oocyst numbers (Blandin et al., 2004; Osta et al., 2004). *A. gambiae* mosquitoes over-expressing TEP1 almost completely aborted the development of the rodent malaria parasite *P. berghei* (Blandin et al., 2004). In *Aedes*

aegypti, transgenic mosquitoes over-expressing Cecropin A and Defensin A showed anti-*Plasmodium* and anti-bacterial responses (Kokoza et al., 2010). *A. stephensi* mosquitoes showed a decrease in *P. falciparum* infection levels when expressing single chain antibodies that inhibited ookinete invasion of the midgut or sporozoite invasion of the salivary gland (Isaacs et al., 2011). *A. stephensi* mosquitoes over-expressing the gene encoding the REL2 transcription factor exhibited increased resistance to P. falciparum through the robust expression of several AMPs and anti-*Plasmodium* effector genes (Dong et al., 2011). Therefore, understanding the interaction of *Plasmodium* parasite in its vector on the molecular and cellular level is important for developing transmission-blocking strategies in the future.

It is not enough to establish a transgenic mosquito; a gene drive mechanism must exist to enable those mosquitoes to spread through the wild type field populations. Transposable elements (TE) are one potential gene drive system where they can colonize other genomes through horizontal transfer and thus transferring the gene through a population. However, one negative aspect is that TEs integrate randomly into a genome. Another gene drive system would be the use of homing endonuclease genes (HEG) which can be engineered to cleave a specific DNA sequence. HEGs can be used to induce genetic sterility in a population by causing sex ration distortion in a population. The expression of the HEG I-Ppol in male gonads to cleave a specific sequence in the X chromosome-linked rDNA locus selectively targeted X-chromosome carrying spermatozoa causing transmission ratio distortion (Windbichler et al., 2008). It was also shown that when transgenic I-Ppo1 male mosquitoes were crossed with wild-type females, they resulted in complete early dominant embryo lethality (Windbichler et al., 2008). Another genetic approach used to reduce mosquito population size was based on release of insects carrying a dominant lethal gene (RIDL) whereby the expression of

a female-specific lethal gene in the adult flight muscles caused a flightless phenotype in *Aedes aegypti* (Fu et al., 2010).

Paratransgenesis refers to the genetic manipulation of specific members of the mosquito microbiota to express molecules toxic against pathogens carried by mosquito vectors of disease. Recently, the interaction of the microbiota with their insect hosts has received increased attention from the perspective of paratransgenesis, which entails engineering symbiotic bacteria to kill pathogens carried by disease vectors. Successful paratransgenesis has been performed in the vector of Chagas disease *Rhodnius prolixus* by engineering its obligate symbiont *Rhodococcus rhodnii* to produce the antimicrobial peptide cecropin A that kills *Trypanosoma cruzi* parasites in the vector (Beard et al., 2002). A similar approach has been adopted to combat *Plasmodium* parasites in the gut of their Anopheline mosquito vectors. *Plasmodium* parasites are the causative agents of malaria which is a great scourge of the world, causing close to 800,000 deaths every year (WHO, 2010). *Pantoea agglomerans*, a common mosquito midgut inhabitant engineered to produce anti-*Plasmodium* effector molecules, inhibited the development of the human malaria parasite *P. falciparum* up to 98% in *An. gambiae*, the major malaria vector in Africa (Wang et al., 2012). Other mosquito gut bacteria are being considered as potential tools for paratransgenesis, in particular the acetic acid bacterium *Asaia sp.* which is common in field populations of several mosquito species (Crotti et al., 2009; Damiani et al., 2010; Favia et al., 2007; Favia et al., 2008). The tractability of *Asaia sp.*is the fact that it is vertically transmitted by colonizing the reproductive organs of both male and female mosquitoes, and hence engineered strains of this bacterium are expected to spread efficiently in mosquito field populations.

Wolbachia are obligate intracellular alpha-proteobacteria that are maternally inherited and which can trigger cytoplasmic incompatibility expressed as embryonic

lethality when infected males are crossed with uninfected female mosquitoes or when crosses occur between individuals infected with different *wolbachia* strains. Interestingly, the introduction of the endosymbiont *Wolbachia* into *Ae. aegypti* compromised mosquito competence for Dengue and Chikungunya viruses (Moreira et al., 2009). *Wolbachia* is particularly attractive because it is maternally inherited and can invade natural populations through cytoplasmic incompatibility with relatively little fitness cost on the host (Frentiu et al., 2014; Hoffmann et al., 2011; Walker et al., 2011).

Moreover the wMelPop strain of *Wolbachia* was used in *Ae. aegypti* and *A. gambiae* (Jin et al., 2009; McMeniman et al., 2009). In *Ae. aegypti*, wMelPop strain of *Wolbachia* reduced the life span by half and showed up-regulation of the mosquito immunity and inhibited the development of filarial nematodes (Kambris et al., 2009). In nature *Wolbachia* does not infect *Anopheles* mosquitoes, but it was shown that wMelPop strain of *Wolbachia* can survive and replicate once injected into *A. gambiae* mosquitoes but the somatic infection was avirulent (Jin et al., 2009).

C. Pattern Recognition Receptors and Recognition of Nonself

Invertebrates are characterized by the absence of an adaptive immune system and they rely merely on the innate immune system. Mosquitoes have several physical barriers to infecting microbes including a chitinous exoskeleton, the peritrophic membrane of the midgut, and the chitinous linings of the trachea. The malaria parasites are able to cross the peritrophic membrane by utilizing a chitinase reaching to the surface of midgut epithelial cells (Shahabuddin et al., 1993). Pathogens including *Plasmodium* parasites that are able to traverse the physical barriers will quickly activate the innate immune system which through the concerted action of its effector molecules will eliminate or control the infection. The innate immune system is triggered through

the recognition of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), peptidoglycans (PGN), and β-1, 3-glucans by soluble or membrane bound pattern recognition receptors (PRRs) (Medzhitov and Janeway, 2002). *A. gambiae* PRRs include leucine-rich repeat proteins (LRRs), C-type lectin-like proteins (CTLs), peptidoglycan recognition proteins (PGRPs), Gram-negative bacteria-binding proteins (GNBPs) also known as β-glucan recognition proteins, Immunoglobulin (Ig) domain proteins and fibrinogen-related proteins (FREPs) (Christophides et al., 2002; Dong and Dimopoulos, 2009; Meister et al., 2009; Warr et al., 2008; Waterhouse et al., 2007). Previous reverse genetic studies identified several PRRs involved in anti-*Plasmodium* defense, including the LRRs LRIM1 and APL1C (Osta et al., 2004; Riehle et al., 2006) GNBP4 (Warr et al., 2008) FBN9 and the Ig-domain protein AgDscam (Dong et al., 2012). The major effect was attributed to LRIM1 and APL1C which circulate as an obligate disulfide-linked heterodimer in the mosquito hemolymph (Povelones et al, 2009). Silencing either LRIM1 or APL1C triggered an increase in the number of parasites that successfully develop to oocysts at the basal part of the midgut epithelium (Osta et al., 2004; Riehle et al., 2006).The LRIM1/APL1C dimer binds to and stabilizes the major anti-*Plasmodium* effector protein TEP1 (thioester-containing proteins1) (Fraiture et al., 2009; Povelones et al., 2009). TEP1 is a complement-like protein with a structure similar to vertebrate C3 but lacks the C345C and anaphylatoxin domains (Levashina et al., 2001). TEP1 is a hallmark of mosquito immunity and will be discussed with the effector mechanisms below.

PGRPs were first isolated from the hemolymph of *Bombyx mori* (Yoshida et al., 1996) and include secreted, transmembrane, and cytoplasmic forms (Werner et al., 2003). Upon bacterial and malarial infection, several *A. gambiae* PGRP genes were

strongly up-regulated (Christophides et al., 2002; Dimopoulos et al., 2000; Dimopoulos et al., 2002). *Anopheles gambiae* genome contains seven members of the PGRP gene family (Christophides et al., 2002). Insect PGRPs can trigger antimicrobial peptide synthesis by activating the major immune signalling pathways Toll pathway (Michel et al., 2001) and Imd (Choe et al., 2002) by initiating the melanization response (Yoshida et al., 1996), or by triggering the phagocytosis of bacteria (Ramet et al., 2002). PGRPs can be divided into two subfamilies: the short form PGRPS subfamily that are extracellular and the long form PGRPL subfamily that are intracellular or transmembrane (Charroux et al., 2009; Waterhouse et al., 2007). The mosquito PGRP genes are regulated through alternative splicing to produce several isoforms that can have different binding specificities and regulation (Christophides et al., 2002). It was shown that PGRP-LC in *A. gambiae* mosquitoes induces the production of antimicrobial peptides against systemic infections with both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli)* bacteria (Meister et al., 2009). PGRP-LC indirectly affects *Plasmodium* parasites by upregulating the expression of anti-bacterial genes that show cross-protection against *Plasmodium*.

PRRs belonging to the family GNBPs were first isolated from *Bombyx mori* and were shown to bind strongly to Gram negative bacteria (Lee et al., 1996). In *Drosophila* GNBP1 binds to LPS and β-1,3-glucan, and together with PGRP-SA they sense Gram-positive bacteria leading to the activation of the Toll pathway (Gobert et al., 2003; Kim et al., 2000). In moth a GNBP homologue was shown to be related to the activation of melanization (Kim et al., 2000). In *A. gambiae* six GNBPs were identified and transcriptomic analysis revealed that GNBPB1 and GNBPA1 were up-regulated by bacterial and malaria parasite infections, respectively (Christophides et al., 2002). Subsequent *in vivo* functional genetic analysis of the six GNBP genes by RNAi revealed

a role for all six GNBPs in defense against bacteria and malaria parasites to different extents. For instance, silencing *GNBPB4* caused an increase in the number of developing *P. berghei* (model rodent parasite) oocysts in mosquito midguts and an increase in mosquito susceptibility to both Gram-positive and Gram-negative bacteria, while *GNBPA2* knockdown (kd) increased mosquito susceptibility to the human malaria parasite *P. falciparum* and to Gram-negative but not Gram-positive systemic bacterial infections (Warr et al., 2008).

On the other hand two CTLs, namely CTL4 and CTLMA2, were identified as potent parasite antagonists. The *A. gambiae* genome contains 23 genes belonging to the CTL. Using comparative genomic analysis, these genes have been classified into different subgroups depending on sequence similarity and domain architecture (Christophides et al., 2002).

Silencing either *CTL4* or *CTLMA2* gene triggered the massive melanization and killing of Plasmodium ookinetes in the basal labyrinth of the mosquito midgut epithelium (Osta et al., 2004), suggesting that these genes act as negative regulators of the potent mosquito melanization response. CTL4 and CTLMA2 form a disulfide-linked heterodimer in the hemolymph which is required for defense against systemic infections with Gram-negative bacteria (Schnitger et al., 2009). The mechanism of action of these CTLs is still unknown and is currently being investigated.

D. Signal Modulation

The recognition of PAMPs by soluble PRRs activates proteolytic cascades comprised mainly of clip-domain serine proteases (CLIPs). Serine protease cascades are composed of several clip-domain serine proteases (CLIPs), in addition to other non-CLIP modular proteases, and are tightly regulated by serpins (SRPNs). The main

function of the cascade is to relay and amplify the signal to downstream transduction pathways or, in certain cases, directly to terminal effector proteins such as phenoloxidase (PO), the key effector molecule in the melanization response. In insects, CLIP protease cascades control several effector responses including the coagulation response in the horseshoe crab (Kawabata et al., 1996), synthesis of antimicrobial peptides through the Toll pathway (Lemaitre and Hoffmann, 2007) and melanization (Kambris et al., 2006; Tong et al., 2005; Volz et al., 2006).

CLIPs are composed of one or more amino-terminal CLIP domains containing cysteine knots, and a carboxy-terminal serine protease domain characterized by HDS residues that function in the proteolytic activity of CLIPs (Waterhouse et al., 2007). Moreover, CLIP domains are believed to play a role in protein-protein interactions, yet there exact function remain to be elucidated. Certain CLIPs have one or more residues in the catalytic site mutated leading to the loss of the proteolytic activity. These are referred to as clip-domain serine protease homologues or SPHs. The role of SPHs in insects has been mainly limited to the triggering of PO activity. The two SPHs, SPH1 and SPH2 of the tobacco hornworm, *Manduca sexta*, play an important role in the prophenoloxidase activation system. These two SPHs act as cofactors for *M. sexta* PAP-1 (prophenoloxidase activating protease), whereby PAP-1 can efficiently activate PPO only in their presence. Since active PAP-1 is fully capable of cleaving PPO, its failure to efficiently do so in the absence of SPHs could be due to its weak interaction with PPO. Thus, SPH1 and SPH2 might be involved in strengthening the interactions between PAP-1 and PPO, by interacting with both proteins and setting PPO at a correct spatial orientation that allows PAP-1 to access it and cleave it at the right site (Yu and Kanost, 2003). Similarly, a masquerade-like SPH of the coleopteran insect *Holotrichia diomphalia*, called PPAFII, has been shown to be indispensable for phenoloxidase

activation. PPAFII acts as a cofactor for the catalytic PPAF-I to cleave PPO into active PO. PPAFII is itself cleaved by the catalytic PPAFIII, suggesting that SPHs, though non-catalytic, still need to be cleaved to become functional (Kim et al., 2002). The same was observed for Manduca SPH1 and SPH2 (Yu and Kanost, 2003). More recently, a non-clip SPH termed SPH3 was shown to play a central role in *Manduca* immunity to Gram-negative bacterium *Photorhabdus luminescens*. Silencing SPH3 severely attenuated the transcription of several antimicrobial effector genes including PPO suggesting a broader role of SPHs in insect immunity (Felfoldi et al., 2011). In *Drosophila*, the SPHs spheroide and sphinx act as regulators of catalytic CLIPs upstream of the Toll pathway to control antimicrobial peptide expression following fungal and Gram-positive bacterial infections (Kambris et al., 2006). In *A. gambiae*, the SPH CLIPA8 was shown to be required for the melanization response to *P. berghei* ookinetes, bacteria and fungi (Schnitger et al., 2007; Volz et al., 2006; Yassine et al., 2012) but its direct involvement in PPO activation is still not validated. On the other hand some CLIPA proteins revealed an opposite role acting as negative regulators of the melanization response (Volz et al., 2006). More recently two A. gambiae SPHs were found to regulate the consumption of the complement-like protein TEP1 on microbial surfaces ((Povelones et al., 2013); Yassine et al, unpublished) hence fine tuning the intensity of the response during systemic infections.

In *A. gambiae*, 54 CLIPs were identified and classified into five subfamilies (A-E). Two subfamilies, A and E, contain mostly non-catalytic CLIPs (SPHs) and the remaining (B, C, D) include mostly CLIPs with catalytic activities (Waterhouse et al., 2007). *A. gambiae* CLIPs were shown to play a role *in vivo* in *P. berghei* ookinete melanization, in *P. berghei* ookinete lysis, and in anti-bacterial defense (Volz et al., 2006; Volz et al., 2005).

SRPNs tightly regulate the activity of serine protease cascades, for example the activation of the Toll pathway in D. melanogaster is related to phersephone; a CLIP protein, and Spn43Ac; a SRPN protein (Levashina et al., 1999; Ligoxygakis et al., 2002). The SRPNs function by inhibiting the activity of serine proteases by covalently binding to the enzyme active site and forming an irreversible serpin-protease complexes (Reichhart et al., 2011; Silverman et al., 2010; Whisstock et al., 2010).

E. Signal Transduction

Signal transduction is an important step in the immune response which relays signals from pattern recognition receptors to downstream effector responsed. In insects, pioneering studies in innate immune signaling were conducted in *Drosophila* because the genetic tools available in this system are unparalleled. Two signaling pathways have been largely implicated in the regulation of *Drosophila* innate immune responses; The Toll and the immune deficiency (Imd). The Toll pathway activates the NF-κB-like transcription factor DIF (Dorsal-related immunity factor) and regulates the immune response towards Gram-positive bacteria and fungi. The Imd pathway activates also an NF-κB-like transcription factor called Relish and regulates the immune response towards Gram-negative bacteria (Lemaitre and Hoffmann, 2007). Comparative genomic analysis between *D. melanogaster* and *A. gambiae* showed that the intracellular compartments of Toll and Imd pathways are conserved, with few exceptions, mainly the absence of a mosquito orthologous of *Drosophila* DIF (Christophides et al., 2002). Rel1 (previously knownas Gambif) (Barillas-Mury et al., 1996) and Rel2 (Meister et al., 2005) are the mosquito NF-κB-like transcription factors orthologous to *Drosophila* Dorsal and Relish, respectively (Christophides et al., 2002). In both species, DIF and Rel1 interact with the negative regulator Cactus that masks the nuclear localization

signal keeping them in the cytosol. Rel2 is also sequestered in the cytosol by an intrinsic inhibitory ankyrin domain similar to that present in *Drosophila* Relish.

The *A. gambiae* Toll/Rel1 and Imd/Rel2 pathways are implicated in defense against the rodent (*Plasmodium berghei*) and human (*Plasmodium falciparum*) malaria parasites (Frolet et al., 2006; Garver et al., 2009; Meister et al., 2005; Mitri et al., 2009), with Rel2 contributing most to anti-*P. falciparum* defense (Garver et al., 2009; Mitri et al., 2009). The potency of the Rel2 pathway in controlling *P. falciparum* infections was further confirmed in transgenic *A. gambiae* mosquitoes expressing blood meal-inducible *Rel*2 in both the midgut and fat body tissues (Dong et al., 2011). The transient activation of this transgene almost completely aborted the developmental cycle of the human malaria parasite in the mosquito. In *A. gambiae*, Toll/Rel1 provides protection against systemic infections with Gram-positive bacteria (Meister et al., 2005), while Imd/Rel2 protects against both Gram-negative and Gram-positive infections (Meister et al., 2009). Both Rel1 and Rel2 control the basal expression levels of several immune proteins including antimicrobial peptides and genes such as *TEP1*, *LRRD7* (leucine rich repeat domain protein 7), and those encoding several FBNs, CLIP (Frolet et al., 2006; Garver et al., 2009). Two isoforms were shown to exist for Rel2 that are produced through alternative splicing: The full length Rel-2F is involved in defense against *Staphylococcus aureus*, while the short one Rel2-S mediates immune defense against *Escherichia coli* (Meister et al., 2005).

Mosquito signal transducers and activators of transcription (STAT) were also shown to be implicated in the defense against *Plasmodium*. *Anopheles gambiae* contains two STAT genes *Ag*STAT-A and *Ag*STAT-B (Christophides et al., 2002). *Ag*STAT-B which was shown to translocate to the nucleus of fat body cells after bacterial infection (Barillas-Mury et al., 1999). AgSTAT-B was shown to regulate *Ag*STAT-A which in

turn controls the transcript levels of Nitric Oxide Synthase (NOS) after *Plasmodium* and bacterial infections. NOS mediated a late-phase response against early *Plasmodium* oocysts hence contributing to parasite killing in the vector. AgSTAT-A also regulated the transient transcriptional upregulation of TEP1 during post-invasion phase of the midgut, which probably serves TEP1 replenishment purposes (Gupta et al., 2009).

More recently, the Jun-N-terminal kinase (JNK) pathway has been also implicated in defense against *Plasmodium* berghei parasites in *A. gambiae* (Garver et al., 2013). The JNK pathway mediated its anti-*Plasmodium* effects by controlling the expression of genes (HPX2 and NOX5) required for parasite nitration during its migration through midgut epithelial cells as well as immune effectot genes TEP1 and FBN9. The relevance of this pathway in mosquito resistance to the human malaria parasite remains to be elucidated.

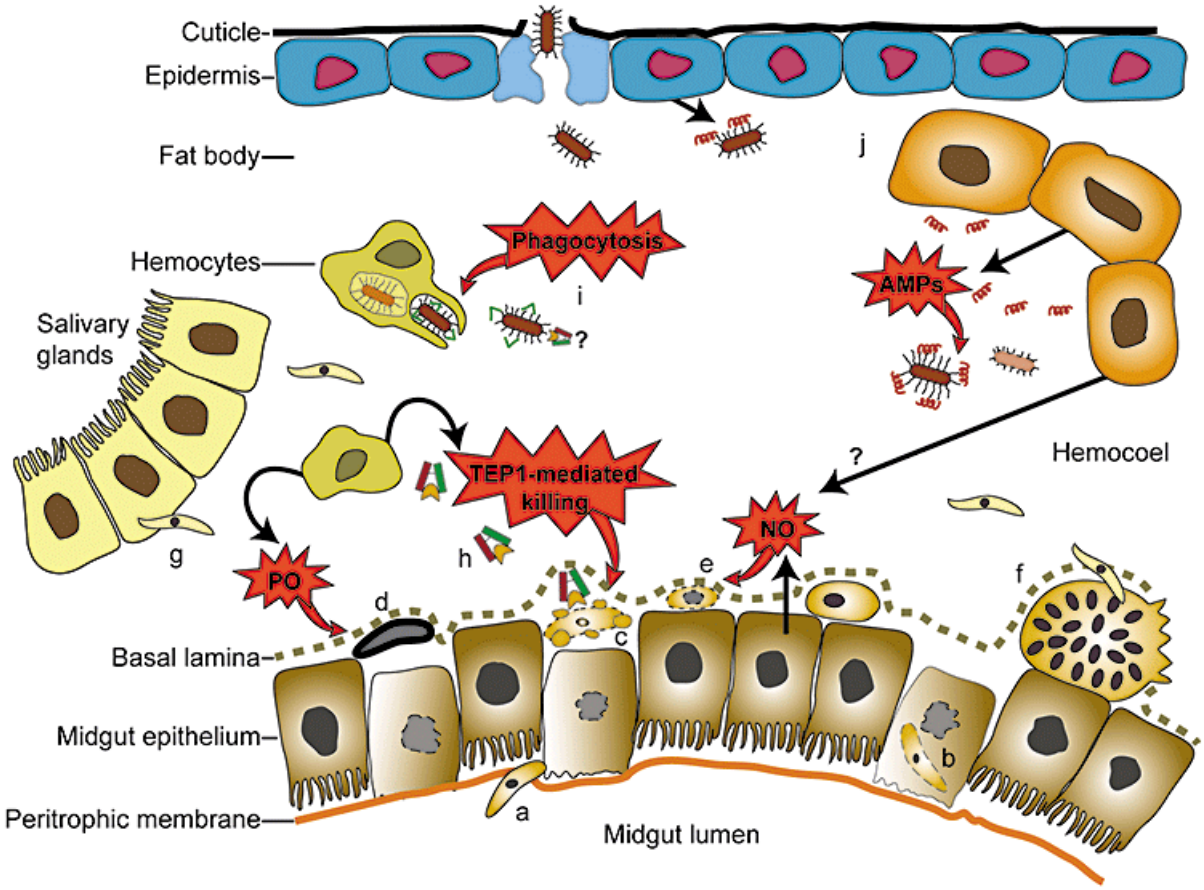

Figure 2. *Anopheles gambiae* immune effector responses. Black arrows indicate the production of different effector molecules by its indicated tissue. (a) and (b) Ookinetes invasion of the midgut epithelium. (c) TEP1-mediated killing of ookinetes in the basal labyrinth. (d) Melanization of ookinetes mediated by PO in the basal labyrinth in certain mosquito genetic background. (e) Nitric oxide produced by epithelial cells and possibly by fat body cells mediates the killing of early oocysts. (f) Escaped early oocysts develop into mature oocysts releasing sporozoites that migrate to (g) and invade the salivary gland epithelium. (h) LRIM1/APL1C/TEP1 complex. (i) Phagocytosis of bacteria in the hemolymph by hemocytes requiring the opsonin TEP1. (j) Production of AMPs systemically by fat body cells and locally by barrier epithelia.
Figure adapted from (Yassine and Osta, 2010).

F. Effector Mechanisms

1. Antimicrobial Peptides (AMPs)

In insects, AMPs are synthesized mainly by the fat body and secreted into the hemolymph. They are mostly small cationic peptides of 15 to 50 amino acids that interact with the cell membrane of its target and disrupt it (Hoffmann, 2003). In *Drosophila*, there are seven different classes of AMPs which include attacins,

cecropins, defensin, diptericins, drosocin, drosomycins, and metchnikowin (Hetru et al., 2003). In *A. gambiae*, AMPs belong to four classes encompassing 11 members. These include four cecropins (CECs), five defensins (DEFs), one gambicin (GAM), and one diptericin (DPT) (Christophides et al., 2002; Waterhouse et al., 2007). CEC1 (Vizioli et al., 2000) and GAM (Vizioli et al., 2001a) have a broad spectrum of activity against Gram-positive, Gram-negative bacteria and *P. berghei* ookinetes. CEC1 also exhibits activity against yeast. Moreover, DEF1 was shown to function against Gram-positive bacteria and some filamentous fungi (Vizioli et al., 2001b). The expression of several of these AMP genes such as *Cecropin1*, *Defensin1* and *Gambicin* seem to be regualted by both Rel1 and Rel2 (Luna et al., 2006).

2. Epithelial Nitration

invasion of midgut epithelial cells by *Plasmodium* ookinetes is not without harm. Invaded cells mount defense responses such as activation the enzyme nitric oxide synthase responsible for the extensive nitration observed in *Plasmodium*-invaded. This response appears to be a two-step process in which induction of NOS expression is followed by increased peroxidase activity (Kumar et al., 2004). Later, it was shown that a heme peroxidase (HPX2) and NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) oxidase 5 (NOX5) together with NOS mediate the nitration of ookinete surface molecules (Oliveira Gde et al., 2012). Importantly, the nitration of the parasite proteins was required for subsequent recognition and killing of *Plasmodium* ookinetes by the hemolymph effector protein TEP1 as they egress from the midgut epithelial cells into the basal labyrinth. Hence, intracellular ookinete nitration seem to render the parasite "visible" to the mosquito complement-like system.

3. Complement-mediated Killing

TEP1 (Frolet et al., 2006), is a complement-like protein homologous to mammalian C3 factor (Levashina et al., 2001) and a major effector molecule against bacterial (Dong et al., 2006a; Levashina et al., 2001; Moita et al., 2005), fungal (Yassine et al., 2012) and *Plasmodium* infections (Blandin et al., 2004; Dong et al., 2006a; Garver et al., 2012; Molina-Cruz et al., 2012; Molina-Cruz et al., 2013). In brief, it is a hallmark of mosquito immunity. TEP1 is a soluble molecule that is secreted into the hemolymph as a full-length form referred to as TEP1-F which is spontaneously cleaved into two fragments that remain tethered together (Levashina et al., 2001). This cleaved form of TEP1, termed TEP1-C, is stabilized in the hemolymph by physically interacting with two LRR proteins, LRIM1 and APL1C (Fraiture et al., 2009; Povelones et al., 2009). TEP1-C is proposed to be the effector form of the molecule since loss of TEP1-C from the hemolymph following *LRIM1* kd or *APL1C* kd is associated with enhanced survival of *Plasmodium* parasites in the mosquito (Osta et al., 2004; Riehle et al., 2006; Riehle et al., 2008), a phenotype similar to that of *TEP1* kd itself (Blandin et al., 2004). TEP1 binds to the malaria parasite P. berghei and facilitates its killing, through lysis or melanization v. It also binds covalently to the surface of bacteria acting as an opsonin triggering their clearance through phagocytosis (Levashina et al., 2001). It was recently shown that the conversion of TEP1-F to TEP1-C following infection is negatively and positively regulated by the SPHs CLIPA2 (Yassine H et. al, submitted) and SPCLIP1 (Povelones et al., 2013), suggesting that the complement-like attack is under tight and complex regulation in the mosquito.

4. Phagocytosis

Phagocytosis in mosquitoes is aided by hemocytes where they recognize, engulf, and destroy invading pathogens and apoptotic bodies. *A. gambiae* mosquitoes have three types of hemocytes; the oenocytoids, the prohemocytes, and the granulocytes. The granulocytes are the most abundant and the only one capable of phagocytosis (Castillo et al., 2006). A semi-quantitative functional genetic screen in *A. gambiae* showed the involvement of 26 genes in phagocytosis, some of which are involved uniquely in the uptake of Gram-negative bacteria or Gram-positive bacteria while and others are required for both processes (Moita et al., 2005). Two different pathways for phagocytosis were identified and are regulated by the intracellular homologs of *Caenorhabditis elegans* CED5 and CED6 that mediate the removal of apoptotic corpses. The CED5 pathway involves the soluble TEP4, the transmembrane β-integrin 2, and the intracellular CED2. The CED6 pathway involves the soluble TEP1, TEP3, and LRIM1 and the transmembrane low-density lipoprotein receptor-related protein (LRP) (Moita et al., 2005). In *A. gambiae*, a soluble complement-like protein TEP1 functions as an opsonin against Gram-negative bacteria *E. coli*. and promotes phagocytosis in mosquito haemocyte-like cell line (Levashina et al., 2001). Moreover, a member of the immunoglobulin superfamily, the mosquito Down syndrome cell adhesion molecule (Dscam), was shown to have a role in phagocytosis against *E. coli* and *S. aureus* in a mosquito cell line (Dong et al., 2006b).

5. Melanization

Melanization, a response unique to arthropods, involves the deposition of melanin on the surface of pathogens. It also plays a role in wound healing. The binding of pattern recognition receptors (PRRs) to pathogen associated molecular patterns (PAMPs) triggers a cascade of serine proteases. The will lead to the conversion of

prophenoloxidase (PPO) into active phenoloxidase and thus leading to melanization (Figure 3). The cascade of serine proteases includes proteins belonging to clip-domain serine proteases (CLIPs) and is tightly regulated by serpins. Melanization was shown to occur against *Plasmodium* ookinetes (Volz et al., 2006), bacteria (Schnitger et al., 2007) and fungi (Yassine et al., 2012). For *Plasmodium* parasites, melanization can serve as a mechanism for direct killing of ookinetes or disposing of dead parasites (Blandin et al., 2004; Shiao et al., 2006; Volz et al., 2006). However, melanization of parasites is rarely seen in field-caught mosquitoes (Niare et al., 2002). As for bacteria, melanization has an important role in modulating tolerance and resistance against bacterial infections in *Drosophila* (Ayres and Schneider, 2008). In *A. gambaie*, it was shown that melanization is not required for the survival of mosquitoes against bacterial infections of *E. coli* and *S. aureus* (Schnitger et al., 2007).

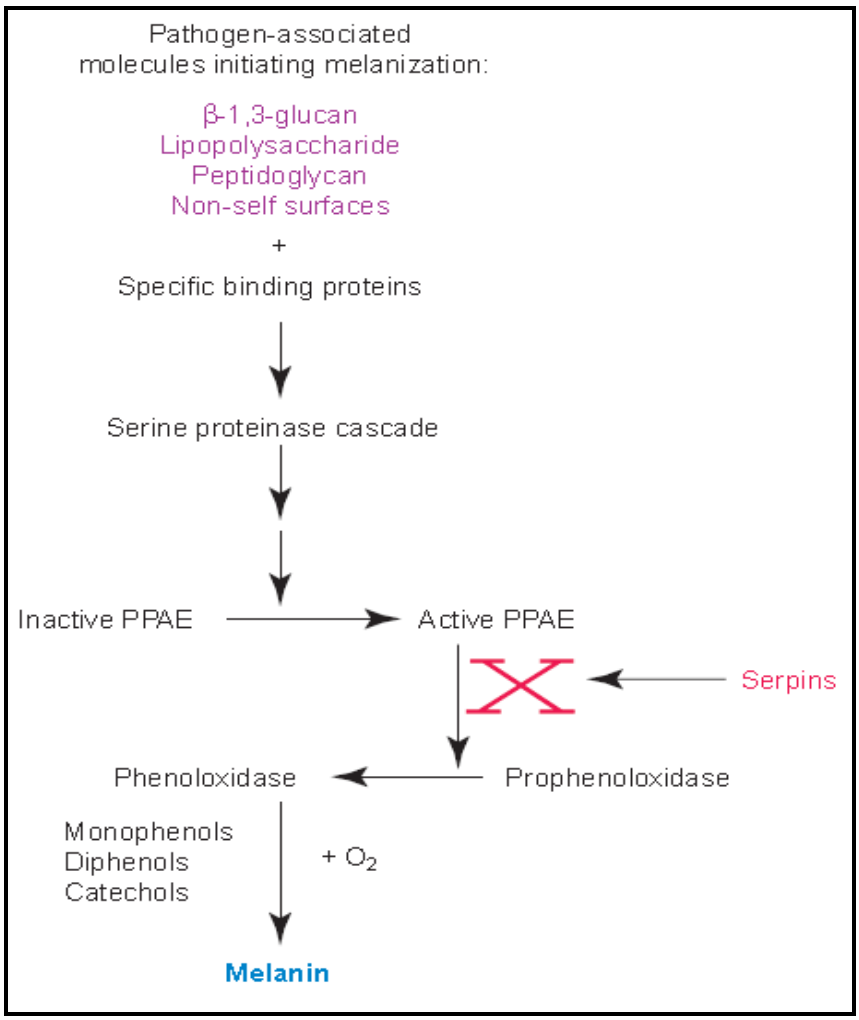

Figure 3. The role of serine proteases and serpins in melanization. Figure adapted from (Christensen et al., 2005).

CHAPTER II

MATERIALS AND METHODS

A. Mosquito Rearing

The mosquito *Anopheles gambiae* G3 strain was used and reared as previously described (Danielli et al., 2000). In concise, mosquitoes were maintained at 27°C with 70% humidity level. They were exposed to a 12h day-night cycle. Adult mosquitoes were fed on 10% sucrose solution and larvae on fish food (Tetramin Flakes ®).

B. Bacterial Strains

The bacterial strains used were *Escherichia coli* (Ampicillin resistant and GFP expressing strain) which was a kind gift from J. J. Ewbank, *Staphylococcus aureus* (Tetracyclin resistant and GFP expressing strain), and *Streptococcus iniae* (ATCC29178T) obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). *E. coli* and *S. aureus* were cultured in Luria-Bertani (LB) medium supplemented with ampicillin (100µg/ml) for *E. coli* and tetracycline (3.3 µg/ml) for *S. aureus. S. iniae* was cultured in Brain Heart Infusion (BHI) medium.

C. Culturing the Bacterial Strains

Overnight bacterial cultures were grown in a shaking incubator at 37°C. Luria-Bertani Broth was used for *E. coli* and *S. aerous*, Brain Heart infusion (BHI) Broth was used for *S. iniae*. The following day, the bacterial pellet was washed four times and resuspended in 1X PBS. The desired concentration was prepared by diluting the stock and measuring the O.D. using a spectrophotometer.

D. Bacterial Challenges by Injection

Adult female mosquitoes (0-3 days) were challenged with bacteria by injecting each mosquito with 69nl of a bacterial suspension having a specific O.D. using a Nanoject II injector (Drummond).

E. Mosquito Survival Assays after Bacterial Infections

Adult mosquitoes (0-1 days) were injected with *LacZ* and *CLIPA8* dsRNAs. After four days they were challenged with bacteria solution by injection. The bacterial solution was prepared from an overnight culture by spinning at 4000 G for 10 minutes and then washing the pellet 4 times with 1X PBS. Later the required O.D. was prepared by using a spectrophotometer. The survival of the mosquitoes was scored daily over a week time basis. For E. coli and S. aureus two separate experiments were done and results were as previous work. Three separate experiments were conducted for S. iniae. The percent survival was calculated using the Kaplan-Meier survival test for each separate experiment of the different treatments. Statistical significance was calculated using the Log-rank test.

F. Gene Silencing by RNA Interference

Double stranded RNAs for LacZ, CLIPA8 were synthesized as previously described (Schnitger et al., 2007). Briefly, gene specific amplicons were amplified by PCR from plasmids using T7-tagged primers listed below.

LacZ-F: TAATACGACTCACTATAGGGAGAATCCGACGGGTTGTTACT

LacZ-R: TAATACGACTCACTATAGGGCACCACGCTCATCGATAATTT

CLIPA8-F: TAATACGACTCACTATAGGGAACAACGAACCCGTAGAATATG

CLIPA8-R: TAATACGACTCACTATAGGGGGTTAGCGCCTCGATACC

The T7-amplicons were purified using Gel Band Purification Kit (GE Healthcare) or the Gene JET Gel Extraction and DNA Cleanup MicroKit and then dsRNA were synthesized using MEGAscript T7 kit (Invitogen) or TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific), according to the manufacturer's instruction. Then the synthesized dsRNA were treated with DNase I. Purification of dsRNA was done using phenol/chloroform and precipitated with propanol. dsRNAs were resuspended in nuclease-free water (GIBCO) at a concentration of 3µg/µl and stored at -20°C.

In vivo gene silencing by RNAi was performed as previously described (Blandin et al., 2002). Mosquitoes were anaesthetized using CO_2 and injected with 69 nl of dsRNA. After 4 days they were challenged with bacteria by injection. The efficiency of gene silencing was previously determined by fellows in the lab using immunoblotting or semi quantitative reverse transcriptase PCR. (semi-quantitative qRT-PCR)

G. Prophenoloxidase Enzymatic Activity

Overnight cultures of E. coli, S. aureus and S. iniae were spun down and the pellet was washed four times with 1X PBS. Bacterial solutions were prepared to a certain O.D. using the spectrophotometer and used for injecting the mosquitoes. After three hours haemolymph was extracted from 80 mosquitoes into ice-cold phosphate-buffered saline (PBS) containing protease inhibitors. Protein quantification was done using Bradford Reagent (Fermentas) and around 5-6 µg of haemolymph proteins were used in each PO enzymatic reaction as previously described (Schnitger et al., 2007). So for each reaction equal amount of protein were prepared in 40 µl PBS containing protease inhibitors and mixed with 120 µl of saturated L,3-4-dihydroxiphenylalanine

solution (LDOPA). Using a Multiskan Ex microplate reader (ThermoLabsystems), absorbance was measured at 492 nm every 10 minutes for a total period of one hour.

H. Western Blotting

Overnight cultures of *E. coli* and *S. iniae* were spun down and the pellet was washed four times with 1X PBS. Bacterial solutions were prepared to a certain O.D. using the spectrophotometer and used for injecting the mosquitoes. 24-30 mosquitoes were used for haemolymph extraction into 20 µl of 1x non-reducing Lane Marker Sample Buffer (Pierce) at the following intervals 0 (uninjected), 2, 5 and 10 hours. The samples were used to study the dynamics of TEP and CLIPA8 cleavage. Only for CLIPA8, the samples were treated with 0.5 µl β-mercaptoethanol for each 12 µl of extracted sample.

The protein samples were separated on 10% SDS-PAGE at 110V for 90 min and then transferred to Immun-Blot Polyvinylidene Fluoride (PVDF) membranes (BioRad) by wet blotting. Membranes were blocked with 3-5 % skimmed milk in PBS-T (1x PBS containing 0.05 % Tween-20) for one hour at room temperature.

Later, membranes were incubated with primary antibody in 3-5 % skimmed milk in PBS-T overnight at 4°C. The primary antibodies used were mouse anti-CLIPA8 monoclonal antibody (1/30) or rabbit anti-TEP1 polyclonal antibody (1/1000) that was affinity purified and rabbit anti-SRPN3 polyclonal antibody (1/1000) as a loading control [or rabbit anti-PPO6 polyclonal antibody (1/2000)].

Membranes were washed three times with PBS-T and then incubated with raddish peroxidase-conjugated secondary antibodies: anti- mouse (1/6000), or anti-rabbit (1/12000) for one hour at room temperature. Then they were washed again three times with PBS-T and overlaid with Luminol reagent (Santa Cruz Biotechnology). Only

ClipA8 Blots were overlaid with Luminol reagent of Amersham ECL Prim Western Blotting Detection Reagent (GE Healthcare). The blots were exposed to Kodak X-ray films for an optional time to detect the proteins.

I. Bacteria Proliferation Assay using Semi-quantitative PCR

Adult mosquitoes (0-1 days) were injected with *LacZ* and *CLIPA8* dsRNAs. After four days they were challenged with bacteria solution by injection. The bacterial solution was prepared from an overnight culture by spinning at 4000 G for 10 minutes and then washing the pellet 4 times with 1X PBS. Later the required O.D. was prepared by using a spectrophotometer. At 24, 48, and 72 hours live mosquitoes were taken for DNA extraction.

DNA extraction was performed using cetyl trimethyl ammonium bromide protocol. Briefly, 10 mosquitoes were homogenized in 200 µl CTAB and then 2 µl of lysozyme (50mg/ml) was added and incubated at 65ºC for 5 minutes. DNA extracted was followed with chloroform and precipitated with 0.7 volumes isopropanol. DNA pellets were suspended in 20 µl of nuclease-free water.

Real time PCR reactions were assembled using QuantiFast SYBR Green PCR Kit (Qiagen) in which 10ng of each DNA samples were loaded and primers for mosquito S7 ribosomal protein gene were used. The program for the real time-PCR was 3 min at 95ºC, 10s at 95ºC, 30 sec at 55ºC, for 39 cycles.

The S7 real time primers were as follows:

S7-F: GTGCGCGAGTTGGAGAAGA

S7-R: ATCGGTTTGGGCAGAATGC

After the real time PCR, samples were normalized to one another for each time point and a PCR was done to quantify *S. iniae* DNA. The *lctO*-specific primers used are:

LOX-1: AAGGGGAAATCGCAAGTGCC

LOX-2 : ATATCTGATTGGGCCGTCTAA

The Program used for PCR reaction was 3 min at 95ºC, 45s at 95ºC, 60 sec at 57ºC, 60 sec at 72 ºC, repeat n cycles, 10 min at 72ºC, end at 4ºC. The number of cycles used was 28, 30 and 32. Quantification of the bands was performed using the BioRad Quantity One software.

CHAPTER III

RESULTS

Most of the studies regarding the antibacterial immune response of utilized model bacteria such as *E. coli* (Gram negative bacteria) and *S. aureus* (Gram positive bacteria), which are most probably not encountered by mosquitoes in their natural habitats and which are not virulent strains. Melanization is an important immune defense of insects however its contribution to immunity, in particular antibacterial immunity, remains controversial in certain insect species like mosquitoes and flies. Using *E. coli* and *S. aureus* as model organisms we have previously shown that melanization in the malaria vector *A. gambiae* is not required for resistance and tolerance of mosquitoes to infections with these bacterial species. Here we revisited the role of melanization in antibacterial immunity using a virulent Gram-positive bacteria *Streptococcus iniae*, which is a known pathogen of marine and fresh water fish. Since we do not know what virulent bacteria exist in the aquatic habitats of mosquito larvae in the field from where adult mosquitoes emerge, we thought that *Streptococcus iniae* may be a good model to study melanization because it is found in water habitats and it is virulent unlike the *S. aureus* strain we use in the lab. *S. iniae* was first isolated from an amazon freshwater dolphin. Moreover, using a broad spectrum of bacterial species in immune challenges may provide a better understanding of the specificity of immune responses in insects as well as identify vector-pathogens combination that would permit the study of the still poorly defined mechanisms of infection tolerance.

A. CLIPA8 Protein is Cleaved by *S. iniae* Infections

CLIPA8 was previously shown to be essential for the mosquito melanization response to *Plasmodium* ookinetes, bacteria (Schnitger et al., 2007) and fungi (Yassine et al., 2012). CLIPA8 is cleaved in the hemolymph of mosquitoes challenged with bacteria (Schnitger et al., 2007). To test whether *S. iniae* triggers the same response in *A. gambiae* mosquitoes, we performed western blot on hemolymph extracted from adult female mosquitoes at 6 hrs post-injection of *S. iniae* and compared CLIPA8 cleavage profile to that following *E. coli* and *S. aureus* injections. Non-injected mosquitoes were used as controls. As expected, in the control lane a full-length CLIPA8 of molecular mass approximately 47 kDa with the absence of the cleaved form of CLIPA8 (CLIPA8-C). Once injected with *E. coli*, *S. aureus*, or *S. iniae*, CLIPA8 cleavage was induced and the cleaved CLIPA8-C form was apparent after all bacterial challeneges (Figure 4). It is worth noting that the more robust cleavage observed after *E. coli* challenge is not due to the inherent ability of *E. coli* to activate a more potent response that that triggered by Gram-positives, but because mosquitoes injected with *E. coli* received much more cfu (colony forming units) than those injected with *S. aureus* and *S. iniae*; In our experience a solution of OD_{600}= 0.4 of *E. coli* contains more cfu/ml with respect to *S. aureus* and *S. iniae* solutions of similar OD.

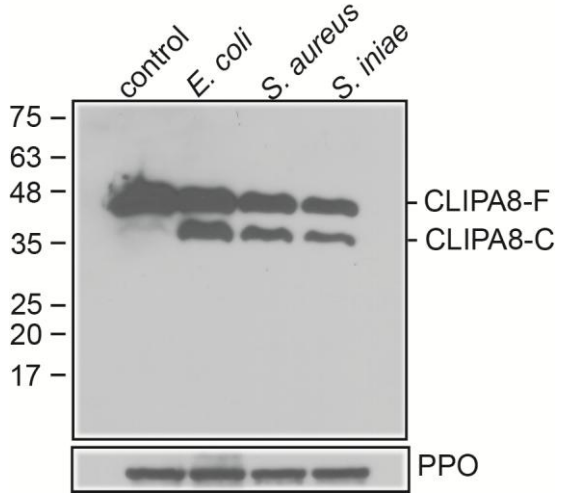

Figure 4. Cleavage of CLIPA8 following mosquito injection with *E. coli*, *S. aureus*, and *S. iniae*. Western blot showing CLIPA8-F and CLIPA8-C in hemolymph collected from wild-type mosquitoes 6 h post-injection with different bacteria species; Gram-negative: *E. coli* (lane 2); Gram-positive: *S. aureus* (lane 3) and *S. iniae* (lane 4). Non-injected mosquitoes were used as controls (lane 1). PPO was used as a control for protein loading.

B. Comparing the Dynamics of CLIPA8 and TEP1 Cleavage Following *S. iniae* and *E. coli* Bacterial Infections

To further characterize the impact of *S. iniae* infection on the evolution of the melanization response in the mosquito compared to an infection with *E. coli*, we followed the dynamics of CLIPA8 cleavage at different time points following bacterial injections. We tried to normalize the numbers of bacteria injected from each specie using their respective survival curves however we did not succeed to inject equal numbers due to variations in the cfu numbers relative to OD readings. In this experiment, we injected mosquitoes with *E. coli* and *S. iniae* bacterial suspensions of equal OD readings (0.8) which corresponded to 2600 *S. iniae* cfu/mosquito and 28000 *E. coli* cfu/mosquito. Western blot analysis of hemolymph samples extracted at 2, 5 and 10 hours after injection of *S. iniae* and *E. coli* revealed distinct cleavage dynamics. *S. iniae* triggered a relatively more potent cleavage of CLIPA8 than *E. coli* at the 5 and 10 h time points, despite the fact that mosquitoes were initially injected with approximately 10 times less *S. iniae* than *E. coli* (Figure 5). Each lane in the blot contained hemolymph extracted from 18 mosquitoes to control for equal protein loading. Anti-PPO antibody was used as an additional loading control.

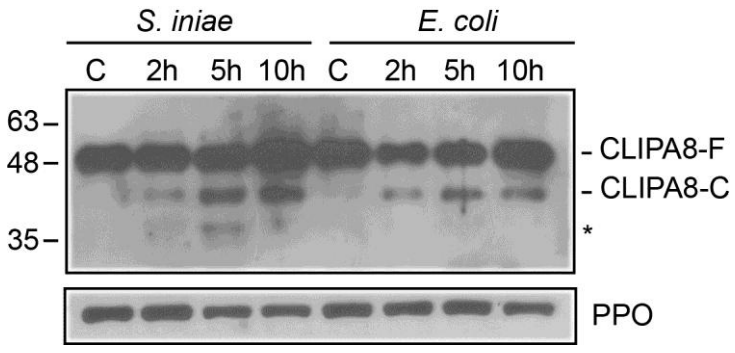

Figure 5. Dynamics of CLIPA8 cleavage by *S. iniae and E. coli*. Western blot analysis showing CLIPA8-F and CLIPA8-C in the hemolymph extracted from adult female *A. gambiae* mosquitoes at the indicated time points post-injection with *S. iniae* (OD_{600}=0.8 representing 2600 CFU/mosquito) and *E. coli* (OD_{600}=0.8 representing 28000 CFU/mosquito) or from non-injected mosquitoes (control). Full-length and cleaved CLIPA8 are labeled CLIPA8-F and CLIPA8-C,

respectively. PPO6 was used as a loading control. The asterisk marks a nonspecific band. h, hours.

TEP1 is another essential protein in the mosquito melanization response to *Plasmodium* ookinetes (Blandin et al., 2004), bacteria (Povelones et al., 2013) and fungi (Yassine et al., 2012). The cleavage of full-length TEP1 form (TEP1-F) to the cleaved active form (TEP1-C) in response to bacterial challenge seems to be a biochemically complex process that is regulated by SPHs (Povelones et al., 2013). However previous assays which monitored TEP1 cleavage dynamics utilized fluorescent *E. coli* bioparticles which are chemically fixed and not live bacteria. Here, we assayed TEP1 cleavage following the injection of live *S. iniae* and compared it to that of live *E. coli.* Hemolymph was extracted at 2, 5 and 10 hours after injection of *S. iniae* and *E. coli*. Each lane in the blot contained hemolymph extracted from 12 mosquitoes to control for equal protein loading. Anti-SRPN3 antibody was used as an additional loading control. The immunoblot showed a difference in the dynamics between *S. iniae* and *E. coli*. Following S. iniae infections, TEP1-F is quickly depleted at the 2 h time points and is replenished at the later time points (Figure 6). TEP1-F depletion is most likely due to its conversion to TEP1-C which attacks bacterial surfaces triggering their lysis, opsonization or melanization. TEP1-F was also quickly depleted from the hemolymph at 2 h post-*E. coli* injections however its levels remained low throughout the later time points, which may indicate that TEP1 is more involved in defense against Gram-negative bacteria as previously suggested (Levashina et al., 2001; Moita et al., 2005).

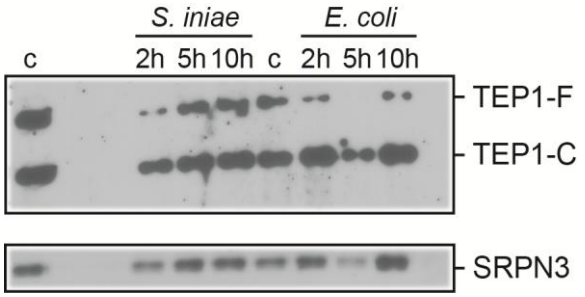

Figure 6. Dynamics of TEP1 cleavage by *S. iniae and E. coli* infections
Western blot analysis showing TEP1-F and TEP1-C in hemolymph samples extracted from adult female *A. gambiae* mosquitoes at various time points post-injection with *S. iniae* (OD_{600}=0.8) and *E. coli* (OD_{600}=0.8) bacteria and from non-injected mosquitoes (control). Full-length and cleaved TEP1 are labeled TEP1-F and TEP1-C, respectively. SRPN3 was used as a loading control.

C. Enzymatic PO activity in the Hemolymph of Mosquitoes Following Injections of *E. coli, S. aureus,* and *S. iniae*

Although CLIPA8 cleavage is a marker of activation of the melanization response, the gold standard for quantifying this response is measuring the enzymatic activity of the enzyme phenoloxidase (PO) in extracted hemolymph samples. PO is the key enzyme in melanin synthesis that catalyzes the rate limiting reaction in that process. Here, we scored hemolymph PO activity following *S. iniae* injections and compared it to that obtained following injections of *E. coli* and *S. aureus*. To that purpose, hemolymph was extracted from 90 mosquitoes per bacterial injection at approximately 3 hours post bacterial injections into mosquitoes. Hemolymph extracted from non-injected mosquito served as control. PO Activity was detected using L-3,4-dihydroxyphenylalanine (L-DOPA) as a substrate. Results of two independent biological replicates revealed that *E. coli* consistently triggered a lower level of PO activity compared to the other Gram positive bacteria *S. aureus* and *S. iniae* although the *E. coli*-injected mosquitoes received more cfu/ injection compared to those injected with the Gram-positive species (Figure 7). When comparing the two Gram-positive bacteria, we noticed that both seem to activate PO to a similar level and that the extent of PO activitation dependent on the numbers of bacteria injected. Hence, whenever the

injected *S. iniae* cfu/mosquito were higher than those for *S. aureus*, PO activity was higher in *S. iniae*-injected mosquitoes, and vice versa (Figure 7).

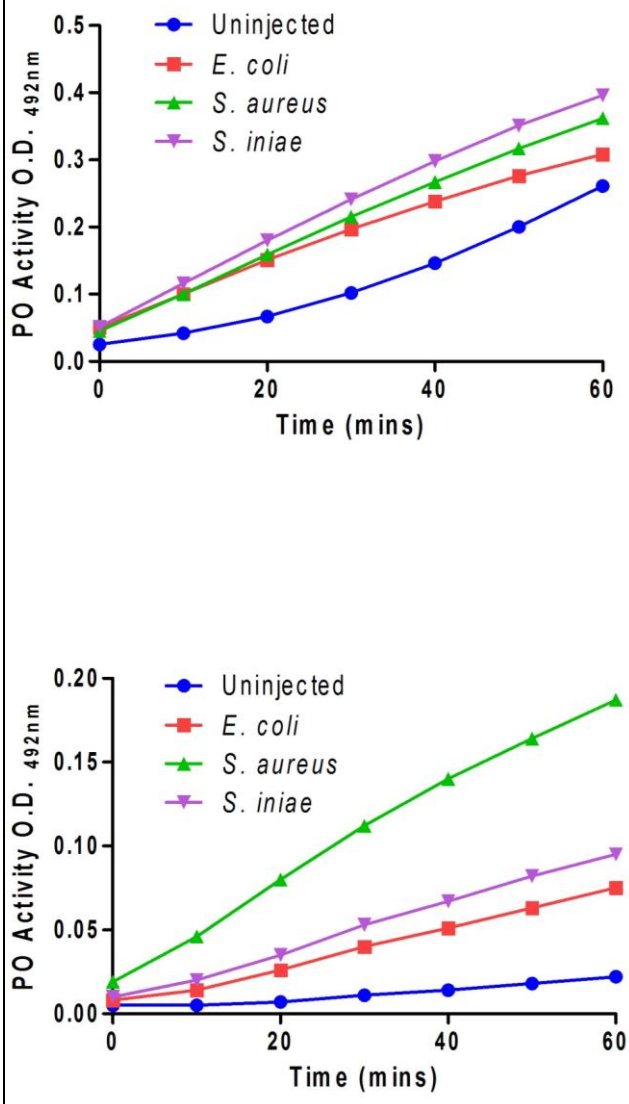

Figure 7. Monitoring PO activities following injections of *E. coli*, *S. aureus*, and *S. iniae*. PO enzymatic activity (detected as absorbance at 492nm, $O.D._{492}$, after conversion of L-3,4-dihydroxypheny lalanine) was measured in hemolymph samples collected from mosquitoes at 3 hrs post injection with *E. coli*, *S. aureus*, and *S. iniae* bacteria and from uninjected mosquitoes (control). Two independent biological replicates are shown. The graphs show PO activity measured at 10-min intervals over a 1 hour period.

D. *CLIPA8* Knockdown Mosquitoes are Susceptible to *S. iniae* Bacterial Infections

It was previously shown that the melanization response is not required for mosquito survival to systemic infections with *E. coli* and *S. aureus* (Schnitger et al., 2007); *CLIPA8* kd mosquitoes failed to activate hemolymph PO yet they were as tolerant and resistant to *E. coli* and *S. aureus* infections as wildtype mosquitoes. Here, we examined whether abolishing melanization by silencing CLIPA8 would alter mosquito susceptibility to *S. iniae* which is supposedly more virulent than *E. coli* and *S. aureus.* To that purpose, *CLIPA8* and *LacZ* kd (control) *A. gambiae*, were challenged with *E. coli, S. aureus* and *S. iniae* four days after mosquito injections with the corresponding dsRNAs. Dead mosquitoes were counted daily over a period of one week. *CLIPA8* kd did not seem to affect mosquito survival to infections with *E. coli* and *S. aureus* (Figure 8) as previously reported (Schnitger et al., 2007); Survival curves of *E. coli* and *S. aureus*-injected mosquitoes were not significantly different between CLIPA8 and *LacZ* kd mosquitoes according to the log-rank test. However, surprisingly, *CLIPA8* kd mosquitoes were significantly more susceptible to *S. iniae* infections than *LacZ* kd controls and that in at least three different biological replicates involving different mosquito batches and *S. iniae* cultures. The compromised mosquito survival observed in *CLIPA8* kd mosquitoes in response to *S. iniae* infections but not *E. coli* or *S. aureus* infections is not due to the initial inoculum size, because in all trials mosquitoes received less *S. iniae* cfu relative to the two other bacterial species.

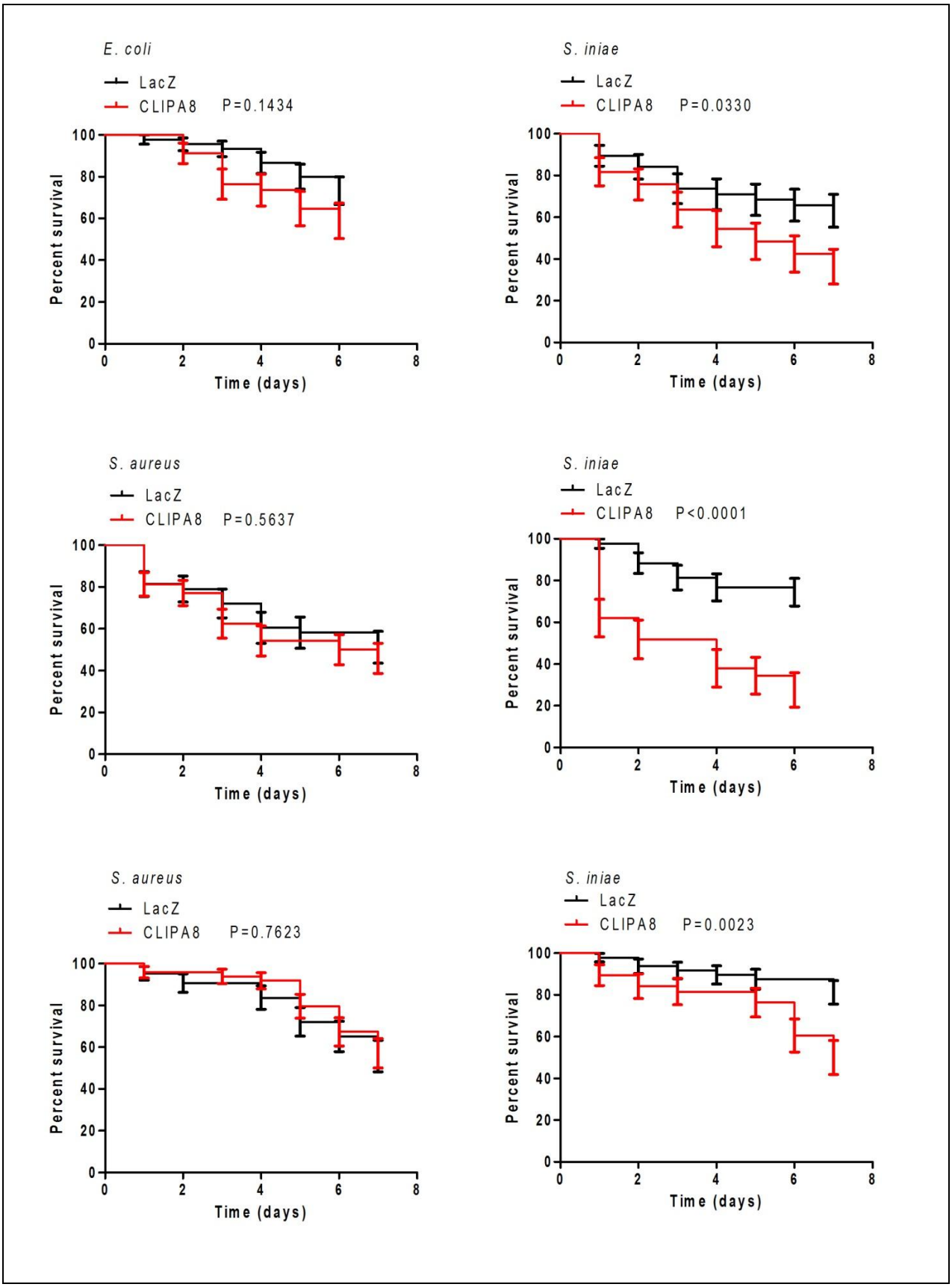

Figure 8. *CLIPA8* kd mosquitoes are more susceptible to *S. iniae* infections than *LacZ* kd controls.

Four days post-injection of ds*LacZ* (control, black color) or ds*CLIPA8* (red color), adult female *A. gambiae* mosquitoes were injected with *E. coli* (50000 CFU/mosquito), *S. aureus* (35000 CFU/mosquito), and *S. iniae* (3500-10000 CFU/mosquito). Dead mosquitoes were counted daily over the indicated period. Graphs represent percent survival as calculated by the Kaplan-Meier method for one representative experiment. Statistical significance was calculated by the log rank test. Survival curves were considered to significantly different if $P<0.05$.

E. Effect of *CLIPA8* Knockdown on the Proliferation of *S. iniae* in Infected Mosquitoes

To determine whether the compromised survival of *CLIPA8* kd mosquitoes to *S. iniae* infections as compared to *LacZ* kd controls is due to increased bacterial proliferation in the former genotype, we performed a semi-quantitative PCR to compare the relative abundance of *S. iniae* genomic DNA between *CLIPA8* and *LacZ* kd mosquitoes. We used *S. iniae* species-specific primers that amplify an 870 bp fragment of the lactate oxidase gene (*lctO*) (Mata et al., 2004) to specifically monitor *S. iniae* abundance in total extracted mosquito DNA. Mosquitoes were first silenced for CLIPA8 or LacZ (control) and after four days they were injected with *S. iniae*. At 24, 48, and 72 hours post-injection whole mosquitoes were grinded and extraction of DNA was performed using the CTAB protocol as described in the Methods section. DNA samples within each time point were first normalized by Real-time qRT-PCR using the mosquito S7 ribosomal protein gene as a reference. Following normalization, a semi-quantitative PCR was performed using the *lctO*-specific primers to determine the relative abundance of *S. iniae* genomic DNA between *CLIPA8* and *LacZ* kd mosquitoes at each time of the indicated point. Quantification of the *lctO* bands were performed using the BioRad Quantity One software. Interestingly, *LacZ* kd mosquitoes exhibited an approximately 2, 3.5 and 4.4-fold increase in *S. iniae* DNA relative to *CLIPA8* kds at 24, 48 and 72 h after *S. iniae* injection, respectively (Figure 9). These results suggest

that the compromised survival of *CLIPA8* kd mosquitoes following *S. iniae* injections relative to controls is not due to increased *S. iniae* proliferation because *CLIPA8* kd mosquitoes consistently harbored less *S. iniae* DNA up to three days post-injection compared to controls, hence pointing to reduced mosquito tolerance to *S. iniae* infections.

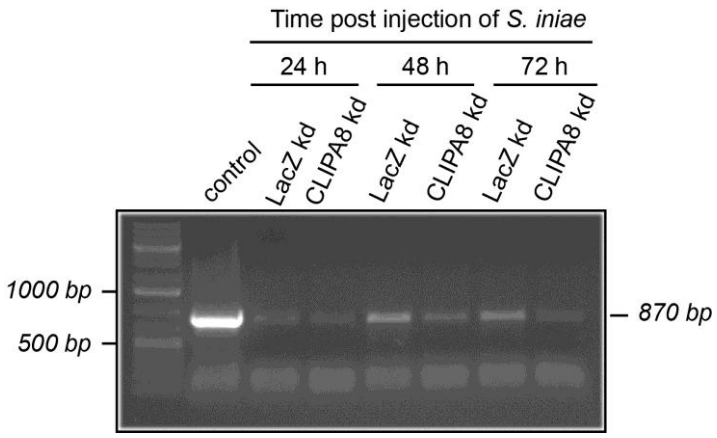

Figure 9. Effect of *CLIPA8* knockdown on the proliferation of *S. iniae*
Four days post-injection of ds*LacZ* (control) or ds*CLIPA8*, adult female *A. gambiae* mosquitoes were injected with *S. iniae* (OD_{600}=1). Mosquitoes were ground and DNA extraction was performed using CTAB method at 24, 48, and 72 hours. Mosquito DNA samples were first normalized by real time PCR using the S7 ribosomal protein gene as reference. Semi-quantitative PCR was performed on normalized samples using *S. iniae*-sepcific primers to the *lctO* gene which amplify an 870 bp fragment of this gene.

CHAPTER IV

DISCUSSION

Melanization is a notable immune response in arthropods which leads to the encapsulation of pathogens in a dense layer of melanin. It requires the cleavage of inactive PPO into active PO by pro-phenoloxidase-acting enzymes belonging to the clip-domain serine protease family and their associated cofactors which are non-catalytic clip domain serine proteases (or serine protease homologs). Melanization also plays an important role in wound healing in insects. Despite the fact that several ultrastructural observations have demonstrated the presence of melanin on bacteria in the hemocoel of different mosquito species (Hernández-Martínez, et al., 2002; Hillyer, et al., 2003a, b; Hillyer, et al., 2004), the contribution of melanization to anti-bacterial defense remain controversial in several insects including mosquitoes. Several reports have linked melanization to insect defense. In the crustacean *Pacifastacus leniusculus*, PO activity is required for defense against the bacterial pathogen *Aeromonas hydrophila*: RNAi-mediated silencing of PO was associated with increased susceptibility to *A. hydrophila* while silencing pacifastin, an inhibitor of the crayfish PO cascade, resulted in increased resistance to the bacterium (Liu et al., 2007). The fact that *Photorhabdus* bacteria pathogenic to *M. sexta* (Eleftherianos et al., 2007), and polydnaviruses carried by female parasitoid wasps (Lu et al., 2008), evolved independent specific strategies to counteract the host melanization response is a further indication of the importance of this response in insect defense.

Previous genetic studies in the model dipteran *Drosophila* revealed that the melanization response does not seem to be critical for survival of flies after bacterial or fungal infections (Leclerc et al., 2006; Tang et al., 2006); rather, melanization seems to

enhance the effectiveness of subsequent immune reactions in the fly by weakening a microbial infection at an early stage (Tang et al., 2006). However, a more recent study, employing a larger panel of bacterial species, demonstrated an important role for this immune process in modulating tolerance as well as resistance of the fly to specific bacterial infections (Ayres and Schneider, 2008). In the malaria vector *A. gambiae* abolishing hemolymph PO activity by silencing the SPH CLIPA8 did not affect mosquito survival after infections with *Escherichia coli* or *Staphylococcus aureus* (Schnitger et al., 2007); In fact neither mosquito tolerance nor resistance were affected after silencing CLIPA8. However, as both *E. coli* and *S. aureus* are not natural pathogens of *A. gambiae* a definite conclusion on the role of mosquito melanization in anti-bacterial defense cannot be reached at present. Here, we extended the analysis of the anti-bacterial role of melanization to *S. iniae*, a gram-positive bacterium prevalent in marine and fresh water habitats (where mosquito larvae prevail) and is known to be pathogenic to fish. We reasoned that *S. iniae* constitutes a better bacterial model than both *E. coli* and *S. aureus* to study the contribution of melanization to immunity because it is a virulent strain and it is prevalent in aquatic habitats where mosquito larvae develop for several days and from where adults emerge. Also the reason we chose a gram-positive bacterium is that gram-positive bacteria are known to trigger a stronger melanotic response in mosquitoes relative to Gram-negatives, which seem to be more rapidly phagocytosed than the former group (Hillyer et al., 2003a, b).

In this study, *S. iniae* injection into adult female mosquitoes triggered a more potent cleavage of CLIPA8, an essential factor of melanization, than *E. coli* injection despite that *S. iniae* cfu injected per mosquito were 10 fold lower than those of *E. coli*. Additionally, *S. iniae* triggered a consistently higher hemolymph PO activity than *E. coli* even in cases where *S. iniae* cfu injected per mosquito were 20 fold less than those

of *E. coli*. Unfortunately we were not able to perform statistical analysis on this data because only two experiments were performed which are shown independently in Figure 8, however the trend in both experiments is similar for all bacterial species tested. Why Gram-positive bacteria are more stronger activators of melanization than Gram-negatives is still not clear. It is possible that Gram-positive bacteria are less prone to efficient lysis by antimicrobial peptides because of their thick and elaborate cell walls which allow them to persist longer in the mosquito hemolymph leading to enhanced stimulation of melanization. It would be interesting in the future to assay whether mutant gram-positive strains with reduced expression of cell wall glycopolymers trigger the same level of PO activity as compared to wildtypes. Mutant strains of *S. aureus* with this characteristic do exist (Atilano et al., 2011) and may be valuable tools to address this question.

TEP1 is the hallmark of mosquito effector responses being involved in the melanization and lysis of *Plasmodium* ookinetes (Blandin et al., 2004), as well as the melanization of bacteria (Povelones et al., 2013) and fungi (Yassine et al., 2012). *S. iniae* infections triggered more or less similar dynamics of TEP1 consumption in immune responses relative to *E. coli*, as in both types of infection TEP1-F was rapidly reduced in the hemolymph at 2h post-infection, reflecting most likely its infection-enhanced cleavage to the effector TEP1-C form. However, TEP1 appeared to be quickly more quickly replenished in *E. coli* infections. This may be due to several factors. First, TEP1 was previously shown to be involved in the phagocytosis of Gram-negative but not Gram-positive bacteria (Moita et al., 2005) which would indicate that Gram-negative infections consume more of TEP1 than those with Gram-positive bacteria, which are more melanized. However, this argument may not be totally convincing since TEP1 is also a key player in melanization which would indicate that it should be

substantially consumed by Gram-positive infections. Another possible explanation for this increased consumption of TEP1-F after *E. coli* infections is that injected mosquitoes most likely received more *E. coli* cfu than *S. iniae*; Although both bacterial suspensions used for injection were at the same OD (0.8), we are almost certain that this OD corresponds to more *E. coli* cells than *S. iniae*, and that according to several experiments performed in our lab, several in the course of this thesis, correlating OD readings with actual cfu. The dynamics of TEP1 consumption by Gram-negative and Gram-positive bacteria require further investigations over a wider temporal range using equal cfu of both Gram types in order to obtain accurate information on TEP1 consumption in anti-bacterial defense. In the course of this thesis work, it has been very difficult to inject equal numbers of bacteria of both Gram-types in all experiments by relying on their growth curves. The variations were always significant, hence we chose to report the numbers of injected bacteria so that results can be correlated with numbers of cfu injected. One way to overcome this obstacle in the future is to use commercial bioparticles of available bacterial strains where appropriate which exhibit a known bacterial concentration. The drawback of these bioparticles is that they are killed bacteria and hence may not elicit the same responses as live ones.

A previous report revealed that abolishing melanization by silencing CLIPA8 in *A. gambiae* did not affect mosquito tolerance to *E. coli* or *S. aureus* since CLIPA8 kd mosquitoes survived as well as wildtypes following either infection. Additionally, *CLIPA8* kd did not affect mosquito resistance because *CLIPA8* kd mosquitoes harbored similar numbers of both bacteria compared to controls. Resistance is directly related to immune mechanisms and indicates the efficiency of clearance of microbes by the host. Tolerance on the other hand is a host defense strategy involving a set of mechanisms, most of which are still poorly characterized, that limit the impact of infection on host

fitness. (Medzhitov et al., 2012). Surprisingly, *CLIPA8* kd mosquitoes were significantly less tolerant to *S. iniae* infections than *LacZ* kd controls as they exhibited a more compromised survival following infection. While, *CLIPA8* kd did not affect mosquito tolerance to *E. coli* or *S. aureus* infections as previously reported (Schnitger et al., 2007). We then assessed whether this reduced tolerance to *S. iniae* in *CLIPA8* kd mosquitoes is due to over-proliferation of this bacterial species in these mosquito genotypes. We were not able to perform viable counts of *S. iniae* in mosquito lysates because the *S. iniae* strain used does not exhibit strong resistance to a given antibiotic which makes its distinction from bacterial members of the gut flora almost impossible. Hence, we resorted to a molecular approach to solve this problem. This entailed using a semi-quantitative PCR with *S. iniae* specific primers to amplify specifically *S. iniae* genomic from total DNA extracted from mosquito lysates. Interestingly, this assay revealed that *CLIPA8* kd mosquitoes contained less *S. iniae* DNA compared to *LacZ* kd controls and that at several days post-infection. This indicates that *CLIPA8* kd mosquitoes are more resistant to *S. iniae* infections than controls but are less tolerant to the infection. To our knowledge, this is the first example in mosquitoes of increased resistance and reduced tolerance to a particular infection in a certain genetic background. The increased resistance observed in *CLIPA8* kd mosquitoes may be explained by the redistribution of energy resources towards more efficient immune effector mechanisms leading to phagocytosis or lysis. Melanization is a complex response at the genetic and biochemical levels which is likely to consume substantial energy from the host, yet may play a secondary role in response to systemic bacterial infections. However, why *CLIPA8* kd mosquitoes are less tolerant to the infection is more difficult to explain, especially that tolerance is still an ill-defined mechanism involving most likely several factors. An evident question that arises here is the

following: Is *S. iniae* causing tissue damage through virulence and somehow melanization is contributing to tissue repair hence minimizing the impact of the infection on the host? Well, *S. iniae* expresses several virulence factors including a polysaccharide capsule that helps escape from phagocytosis and pore-forming toxin that mediates autolysis of eukaryotic cells (Buchanan et al., 2008). A whole genome transcriptomic approach comparing gene expression profiles between *CLIPA8* and *LacZ* kd mosquitoes following *S. iniae* infection may allow the identification of candidate genes that could help explain the nature of tolerance mechanisms in insects.

In conclusion, our study shows that *S. iniae* bacterial infections in *CLIPA8*kd *A. gambiae* mosquitoes lead to reduced tolerance and increased resistance to infection. Hence, it seems that melanization plays a role against bacterial infections by affecting tolerance and resistance in a species-dependent way. Thus, it is important that in future studies addressing the contribution of this immune response, a larger panel of bacterial species be used in order to try to identify potential bacterial features that could probably explain why melanization is needed in defense against only certain bacteria.

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