AMERICAN UNIVERSITY OF BEIRUT

THE ROLE OF CLIP-DOMAIN SERINE PROTEASE HOMOLOGS IN *A. GAMBIAE* IMMUNE RESPONSES TO SYSTEMIC INFECTIONS

by
LAYLA YEHYA KAMAREDDINE

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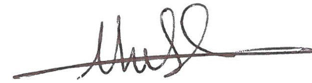
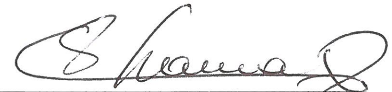


Dr. Mike Osta, Associate Professor — Advisor
Biology

Dr. George Christophides, Professor — Member of Committee
Department of Life Sciences, Imperial College London

Dr. Sawsan Kreydiyyeh, Professor — Member of Committee
Biology



Dr. Souleima Chamat, Professor — Member of Committee
Faculty of Medicine, Lebanese University

Dr. Zakaria Kambris, Assistant Professor — Member of Committee
Biology

Date of dissertation defense: January 8th, 2016

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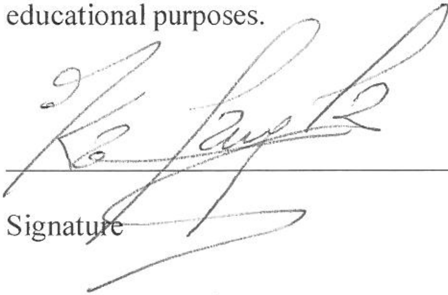

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As I am writing these lines, I flip back the page to the past six years of my life…. To the time I first joined Dr. Osta's lab as a graduate student,to the time I completed my masters degree and decided to continue my doctoral studies, to the last experiment I did on that same bench that I've worked on for years and years…..…… Leaving this group is not going to be that smooth on me, as I've been used to it over these years; yet, every finish is a new beginning by itself …..I thank God for all I've achieved and ask him to grant me the strength to continue the way!

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Towards a new start as "The future belongs to those who believe in the beauty of their dreams!"

AN ABSTRACT OF THE DISSERTATION OF

Layla Yehya Kamareddine for Doctor of Philosophy
Major: Cell and Molecular Biology

Title: The role of clip-domain serine protease homologs in *A. gambiae* immune responses to systemic infections

Malaria is one of the most devastating mosquito-borne infectious diseases of humans in the tropical and subtropical regions. It is caused by parasites of the genus *Plasmodium* which develop through several stages inside the mosquito vector before being transmitted into the human host. Functional genetic studies in *A. gambiae*, the major malaria vector in Sub-Saharan Africa, where mortalities from malaria are the highest, showed that the mosquito is not a passive vector for the parasite; rather, the parasite suffers dramatic reduction in numbers in its transitional development from the ookinete to the oocyst stage. These losses have been largely attributed to the mosquito immune response, and several immunity genes involved in that process have been identified. Initial efforts to study mosquito immunity were directed towards the malaria parasite, being an important pathogen to mankind. However, the fact that several immune effector responses that efficiently kill *Plasmodium* parasites, particularly in the ookinete stages, were are also found to be effective against bacteria, and that the midgut microbiota of the mosquito has significant impact on the survival of parasites, broadened the spectrum of microorganisms used in mosquito immunity studies. The general belief that emerged from these studies collectively is that bacteria and fungi constitute most likely the main pressure on the mosquito immune system in the field rather than malaria parasites which are carried by a small percentage of mosquitoes that do not exceed 2% in endemic regions during peak seasons of transmission. Hence, the use of bacteria and fungi as model pathogens in mosquito immunity studies offer several advantages: First, these microorganisms are more tractable than malaria parasites in several aspects which help deciphering certain immune mechanisms at the molecular level in a manner that would not be possible using the parasite. Second, they might be more relevant than malaria parasites to the understanding of mosquito immunity since bacteria and fungi constitute the main threat to the larval stages in their aquatic habitats. Therefore, in the context of this thesis I focused particularly on understanding mosquito immunity to fungal infections, a field that has been poorly investigated.

I first established a reliable method to infect mosquitoes with spores of *B. bassiana* using tarsal contact in a way that mimics natural infections in the field. I tested the sensitivity of my method by comparing *A. gambiae* susceptibility to wild-type and a

virulent transgenic-TMOF-expressing *Beauveria bassiana* fungus using LD50 (lethal doze 50) and LT50 (lethal time 50) measurements. I next used this infection to address the contribution of the mosquito melanization response to anti-fungal defense and highlight the role of the key immunity genes in that process. Finally, I focused on understanding the role of key immunity genes belonging to the clip domain serine protease (CLIPs) family in mosquito systemic infections with fungi and bacteria. My target gene was CLIPA2, a non-catalytic CLIP, which was previously shown to act as a negative regulator of the mosquito melanization response to *Plasmodium* parasites. My data revealed that CLIPA2 acts as a key negative regulator of the mosquito complement like protein TEP1, considered as the hallmark effector molecule in the mosquito immune system. Silencing CLIPA2 increased mosquito resistance and endurance to fungal and bacterial infections, providing novel insight into the importance of TEP1 in anti-fungal immunity. I next identified the broad spectrum of hemolymph proteins that interact with CLIPA2 in fungal infected mosquitoes using co-immunoprecipitation followed by mass spectrometry. Functional genetic analysis of candidates genes identified Apolipophorin II/I as a novel negative regulator of mosquito TEP1, exhibiting a similar RNA interference (RNAi) phenotype as that of *CLIPA2*. Interestingly, whereas CLIPA2 regulates TEP1 on the protein level, Apo II/I controls the expression of TEP1 gene in a c-Jun N-terminal kinase (JNK) pathway dependent manner, indicating that mosquito complement is subject to complex regulation involving different classes of proteins possibly to avoid an exaggerated immune response that may impose a fitness cost upon the host.

Collectively, the results of my PhD work reveal novel insights into mosquito immunity against systemic infections, particularly with entomopathogenic fungi, and identified distinct mechanisms that seem to act in concert to control the mosquito TEP1-mediated immune response during these infections. They also highlight the existence of a functional interplay between immune gene regulation and lipid metabolism. These results also provide a better mechanistic understanding of some of the RNAi phenotypes observed in mosquitoes infected with malaria parasites due to the broad spectrum of several mosquito immune effector responses especially those mediated by TEP1.

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ABBREVIATIONS

Ae. aegypti	*Aedes aegypti*
A. gambiae	*Anopheles gambiae*
AMP	Anti-microbial peptide
APL1C	*Anopheles Plamodium*-responsive leucine-rich repeat protein 1C
Apo II/I	Apolipophorin II/I
Asp	Aspartic acid
AZ	Organelle-free actin zone
B. bassiana	*Beauveria bassiana*
B. mori	*Bombyx mori*
BINT	β-intergin
BSA	Bovine serum albumin
Cdc2	Cell division cycle 42
cDNA	Complementary DNA
CEC	Cecropin
CED	*C. elegans* cell death abnormal gene
CFU	Colony forming unit
CID	Collision Induced Dissociation
clip-SPHs	Clip-domain serine protease homologs
CO_2	Carbon dioxide
CRASP	Complement regulator-acquiring surface protein
CRD	Carbohydrate recognition domain
CTL	C-type lectin

CTLD	C-type lectin-like domain
DAG	Diacylglycerol
D. melanogaster	*Drosophila melanogaster*
DCE	Dopachrome conversion enzyme
ddH_2O	Double distilled water
dkd	Double knock down
DEF	Defensin
DHI	5,6-dihydroxyindole
DIF	Dorsal-related immunity factor
Dopa	3,4-dihydroxyphenylalanin
DPT	Diptericin
Dscam	Down syndrome cell adhesion molecule
dsRNA	Double-stranded RNA
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FBN	Fibrinogen domain immunolectin
FBS	Fetal bovine serum
FREP	Fibrinogen-related protein
Fz2	Frizzled-2
GAM	Gambicin
GFP	Green fluorescent protein
GNBP	Gram-negative binding protein
GPI	Glycosylphosphatidylinositol

HCL	Hydrochloric acid
H. diomphalia	*Holotrichia diomphalia*
His	Histidine
HP	Hemolymph protease
HPX	Heme peroxidase
IACUC	Institute Animal Care and Use committee
Ig	Immunoglobulin
IKK	IκB kinase
Imd	Immune deficiency
IRS	Indoor residual spraying
IP	Immunoprecipitation
IPM	Integrated pest management
ITN	Insecticide-treated net
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
Kd	knockdown
LB	Luria Broth
LD50	Lethal doze 50
LDLR	Low density lipoprotein receptors
LT50	lethal Time 50
LIC	Ligase-independent cloning
Lp	Lipophorin
LpR	Lipophorin receptor
LPS	Lipopolysaccharide

LRIM1	Leucine-rich repeat immune protein 1
LRP	Lipoprotein receptor-related protein
LRR	Leucine-rich repeat
M. sexta	*Manduca sexta*
MASP	MBL-associated serine protease
MAC	Membrane attack complex
MBL	Mannose-binding lectin
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
Nim	Nimrod
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOS	Nitric oxide synthase
NOX5	NADPH oxidase 5
O.D.	Optical density
O.N.	Overnight
PAH	Phenylalanine hydroxylase
PAMP	Pathogen-associated molecular pattern
PAP	Prophenoloxidase-activating proteinase
P. berghei	*Plasmodium berghei*
P. falciparum	*Plasmodium falciparum*
P. gallinaceum	*Plasmodium gallinaceum*
P.Knowlesi	*Plasmodium Knowlesi*
P.malariae	*Plasmodium malariae*

P. ovale	*Plasmodium ovale*
P. vivax	*Plasmodium vivax*
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline-Tween
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PGRP	Peptidoglycan recognition protein
PO	Phenoloxidase
PPAE	Prophenoloxidase-activating enzyme
PPAF	Prophenoloxidase-activating factor
Ppm	Parts per million
PPO	Prophenoloxidase
PRR	Pattern recognition receptor
psh	Persephone
QTL	Quantitative trait loci
RCL	Reactive center Loop
RNAi	RNA interference
Rpm	Round per minute
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
S. aureus	*Staphylococcus aureus*
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
Ser	Serine

SES	Sub epithelial space
Sf9	*Spodoptera frugiperda* 9
SFM	Serum free medium
SP	Serine protease
SPE	Spz processing enzyme
SPH	Serine protease homolog
Spz	Spaetzle
SRPN or spn	Serine protease inhibitor or serpin
STAT	Signal transducers and activators of transcription
TEP	Thioester-containing protein
TLR	Toll-like receptor
TMOF	Trypsin modulating oostatic factor
Tris	Trisaminomethane
UV	Ultraviolet
Vg	Vitellogenin
WHO	World Health Organization
WT	Wildtype

CHAPTER I

INTRODUCTION

Malaria is one of the most devastating mosquito-borne infectious diseases of humans caused by parasites of the genus *Plasmodium*. In 2014, the world health organization reported around 198 million cases of malaria; of those, around 584 000 died of the disease, mainly children below the age of five (WHO, 2014). With such high mortality and morbidity rates, the disease exerts serious social and economical burdens on humans, mainly those living in poverty. Malaria transmission is ongoing in 97 countries worldwide; however, the burden is heaviest in Africa, particularly in the sub-Saharan region were poverty, difficult access to efficient health services, poor infrastructure and presence of highly anthropophilic vectors, such as *Anopheles gambiae*, aggravate the problem (WHO, 2014).

Despite being potent vectors for malaria transmission, Anopheline mosquitoes are not considered passive hosts for *Plasmodium* parasites, rather these parasites encounter dramatic losses during their life cycle inside the mosquito especially in the transition from the ookinete to oocyst stage, whereby approximately 80% of invading parasites are killed by immune effector responses elicited by the mosquito (Blandin and Levashina, 2004a). Anopheline vectors of malaria, like all other invertebrates, depend only on their innate immune system to fight pathogens. This system is typically activated when pattern recognition receptors (PRRs) recognize conserved pathogen associated molecular patterns (PAMPs) and trigger a downstream signal modulation cascade, composed mostly of clip domain serine proteases (CLIPs), culminating with the activation of several immune effector responses that act concertedly to eliminate the pathogen. CLIPs are key factors that regulate several important immune effectors reactions in mosquitoes, and other insects in general, such as coagulation, complement

amplification, antimicrobial peptide synthesis and melanization. In this thesis, we aim to provide new molecular and functional insights into the role of these CLIPs in the malaria vector *Anopheles gambiae* using several classes of model pathogens including malaria parasites (*Plasmodium berghei*), bacteria (*Escherichia coli* and *Staphylococcus aureus*) and fungi (*Beauveria bassiana*). The importance of the work is twofolds. First, understanding the molecular interactions between mosquito vectors and their parasites may lead to the design of novel strategies that may contribute to reducing the transmission of malaria; For instance, identification of key immunity proteins that are hijacked or neutralized by specific parasite surface proteins for immune evasion may lead to the design of transmission blocking vaccines. Novel strategies for combating malaria are especially important since no efficient vaccine exists yet for this disease and drugs available on the market may lose their effectiveness due to the emergence of drug-resistant parasites (Fidock, *et al.*, 2008). Furthermore, vector control strategies which have shown significant success over the last decade such as the use of insecticide-treated bednets, indoor residual spraying and larvicides, may be compromised in the future due to the rapid spread of insecticide resistant mosquitoes (Reviewed in Kamareddine, 2012). On the other hand, addressing the molecular mechanisms underlying the function of mosquito CLIPs is expected to contribute new knowledge to insect innate immunity and more importantly understand the different "strategies" that shape the *modus operandi* of the innate immune response.

A. The malaria parasite life cycle within the mosquito vector

The etiologic agents of malaria are the Apicomplexan parasites of the genus *Plasmodium* that are transmitted to man through the bite of few female mosquito species belonging to the genus *Anopheles*. Among different *Plasmodia*, *P.malariae, P.*

ovale, P. falciparum, P. vivax, and recently *P. knowlesi* were shown to infect humans (Chen, *et al*., 2008). Other *plasmodium* species reside in non-human hosts namely old and new world existing monkeys, rodents and chickens. In addition to *Anopheles*, *Culex* and *Aedes* mosquito species also transmit different types of the *Plasmodium* parasite (Mehlhorn and Piekarski, 2002). Before being transmitted to the vertebrate host, the *Plasmodium* parasite undergoes a complex life cycle within its mosquito vector (Chen, *et al*., 2008). The sexual development of the parasites initiates few minutes following the intake of an infectious blood meal by the mosquito. Blood ingestion promotes physical and chemical changes inside the mosquito gut quickly triggering the gametogenesis of female (macrogamete) and male (microgamete) gametocytes (Billker, *et al*., 1997; Billker, *et al*., 1998). Following fertilization, a zygote is formed which differentiates eventually into a motile ookinete that penetrates the peritrophic membrane and invades the midgut epithelium at approximately 12 hours post infection. Invading ookinetes arrest at the basal labyrinth of the midgut epithelium where they mature into occysts. Oocysts further mature and enlarge in size within the next 10 days and undergo numerous rounds of mitotic divisions to produce thousands of haploid sporozoites. Approximately two weeks post infection, the mature oocysts start to rupture and release the sporozoites into the hemocoel of the mosquito, from which they migrate to and invade the salivary glands. The infective sporozoites further mature for few more days in the lumen, and thus become ready to infect a new host upon the ingestion of a new blood meal (figure 1). The whole developmental cycle of the parasite in the vector takes around 3 weeks, the time needed for mosquitoes to become infectious to humans.

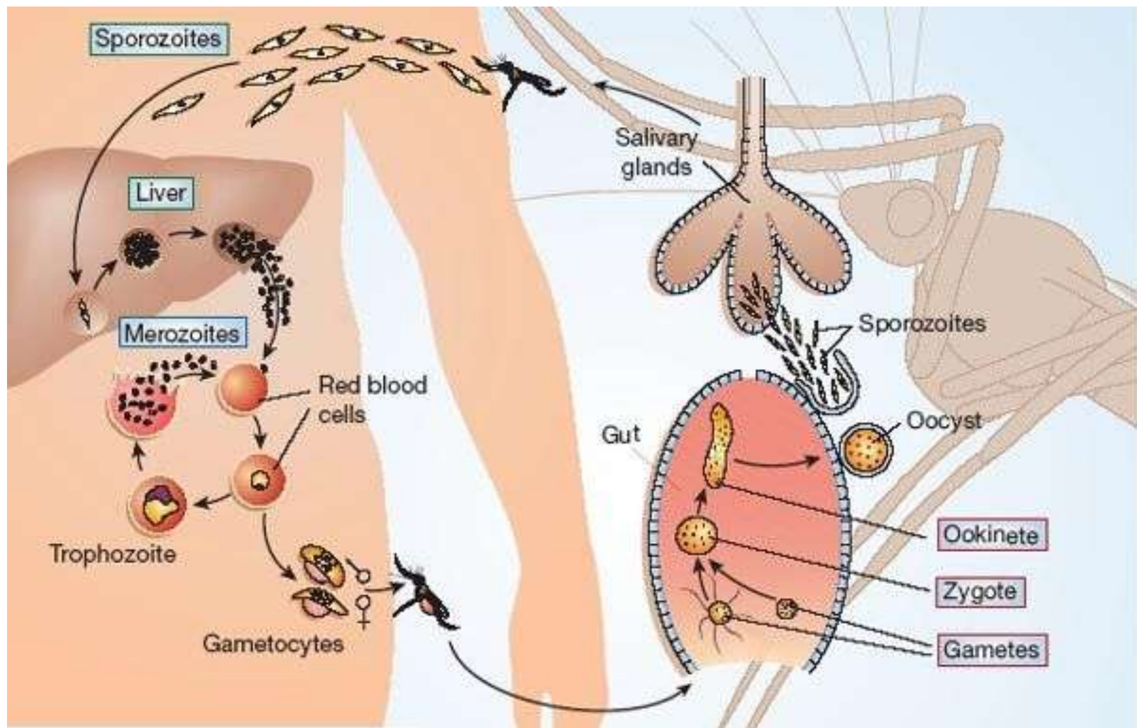


Figure 1. The life cycle of the *Plasmodium* parasite. The *Anopheles gambiae* releases the parasite sporozoites from its salivary gland into the bloodstream of the infected host. These sporozoites then develop in the human liver during the pre-erythrocytic stage, producing merozoites that further travels in the blood stream to infect red blood cells in the erythrocytic stage. At this stage, merozoites mature into rings, trophozoites, and schizonts, producing more merozoites to re-infect other new red blood cells. Occasionally, some merozoites differentiate into female and male gametocytes that mature into gametes and undergo fertilization to form a zygote that develops into a motile ookinete. These ookinetes then traverse the midgut epithelium, reach the basal lamina, develop into oocysts, and undergo numerous rounds of mitotic division to produce thousands of sporozoites which are released into the mosquito hemocoel and invade the salivary glands. (Adapted from Ménard, 2005).

B. Mosquito innate immunity

Like all invertebrates, mosquitoes lack adaptive immunity and depend soley on their innate immune system, with both its cellular and humoral branches, to fight against invading pathogens such as parasites, bacteria, viruses and fungi. In insects, the first line of defense encountered by pathogens is the cuticle of the exoskeleton, the tracheal wall, and the midgut epithelium (Reviewed in Christensen, *et al*., 2005). When microorganisms break through these barriers, they either encounter local defense by

epithelia or systemic defense launched by hemocytes (blood cells) and the fat body (analogous to mammalian liver). Hemocytes and fat body cells release immune effector molecules into the hemolymph such as anti-microbial peptides (AMPs), complement proteins, and prophenol oxidase (PPO), among others. The main effector mechanisms in insect's innate immunity include the production of AMPs, coagulation, agglutination, phagocytosis, nodulation (mainly in large insects), pathogen encapsulation, and melanization (Reviewed in Osta and Yassine, 2010; Reviewed in Christensen, *et al*., 2005; Jiang, *et al*., 2011, An, *et al*., 2011) (figure 2). Noticeably, the relative contribution of these aforementioned effector mechanisms to insect immunity is likely to vary depending on the class of the pathogen and the insect species invaded.

The establishment of efficient molecular and genetic tools has led to remarkable advances in the field of mosquito immunity. Sequencing the *Plasmodium* parasite and the *Anopheles gambiae* genome (Holt, *et al*., 2002), the use of RNA interference (RNAi) to study gene function (Blandin, *et al*., 2002), and DNA microarrays for genome expression profiling (Grossman, *et al*., 2001; Dimopolous, *et al*., 2002) were among the most important established strategies that contributed significantly to the understanding of mosquito interactions with *Plasmodium* as well as with other classes of microbes.

The interest in studying *A. gambiae* immunity was fueled originally by the genetically selected refractory R strain L-35 which melanized several species of *Plasmodium* including some *P. falciparum* strains following midgut invasion (Collins, *et al*., 1986). Genetic mapping of these R strains (Zheng, *et al*., 1997; Zheng, *et al*., 2003) along with other field-caught *A. gambiae* mosquitoes (Niare, *et al*., 2002; Riehle, *et al*., 2006) lead to the recognition of many quantitative trait loci (QTLs) implicated in this mosquito refractoriness to *Plasmodium* parasites, and to the identification of novel

candidate genes such as APL1 that play important roles in this resistance phenotype. (Riehle, *et al*., 2006). Systemic functional genetic analysis of putative immunity genes by RNAi as well as candidate gene identification by high throughout put expression studies identified several key immune factors belonging to pattern recognition receptors, signal modulation, and signal transduction (Reviewed in Sim, *et al*., 2014). However, the mechanism of action of several immune factors remains largely unknown and is partly addressed in the context of my thesis.

In the 1990's, gene expression studies in *A. gambiae* based on differential display showed local and systemic up-regulations of several immune markers in the mosquito abdomen in response to ookinetes invading the mosquito midgut and sporozoites invading the salivary gland epithelium. Collectively, these studies proposed that *Anopheles* immune system is activated by *Plasmodium* and suggested that immune signaling occurs between tissues (Dimopoulos, *et al*., 1997; 1998; Richman, *et al*., 1997). The use of microarrays for genome-wide expression analysis later confirmed that *Plasmodium* infection indeed up-regulates several immunity genes, as well as other classes of genes like those involved in cytoskeletal rearrangement (Mendes, *et al*., 2008), and a very similar up-regulation also exists upon bacterial infections (Christophides, *et al*., 2002; Dimopoulos, *et al*., 2002). Genome wide profiling showed that *P. falciparum* and *P. berghei* infections exhibit different effects on the mosquito transcriptome, indicating that the defense against these two *Plasmodium* species involves the activity of both universal and *Plasmodium*-species specific antimicrobial factors (Dong, *et al*., 2006). Taken together, these results led to the classification of immune genes under the different immune reactions known to be elucidated by mosquitoes namely complement-like attack, phagocytosis, melanization, among others. While most studies on immunity initially focused on *Plasmodium* and bacteria, and later

on viruses, responses to entomopathogenic fungi remain poorly understood although fungi might be a greater threat to mosquitoes in their microenvironment as compared to bacteria and parasites, because they can naturally infect mosquitoes through tarsal contact without any need of ingestion. Some aspects of *Anopheles* immunity to *B.bassiana* infection will be addressed in one part of my thesis.

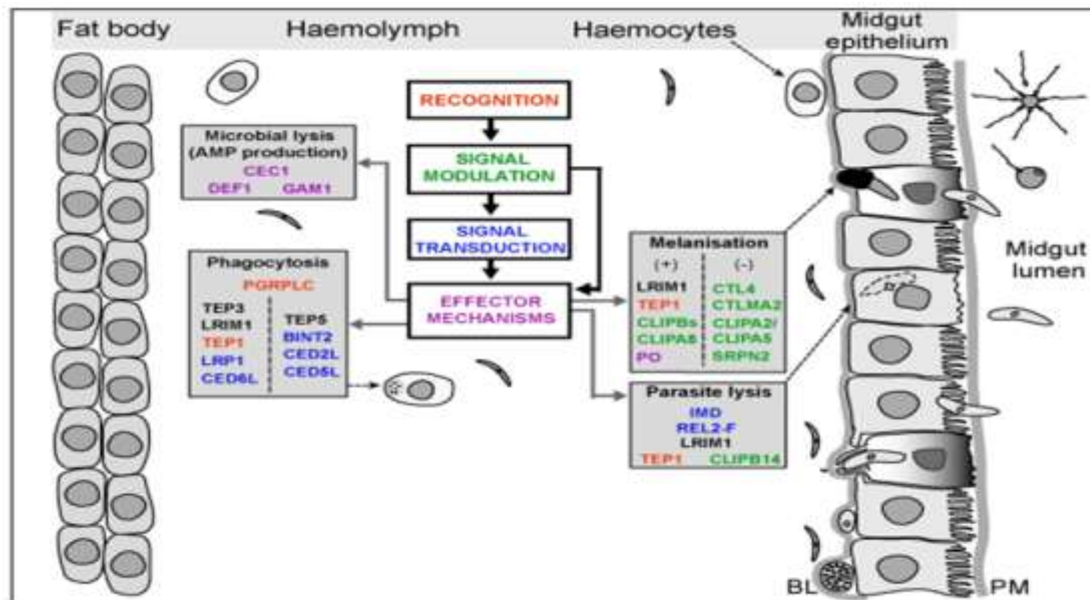


Figure 2. Schematic representation of the conventional immune responses in mosquitoes. Pattern recognition receptors recognize the invading parasite, promoting signal modulation that either directly activates different immune effector mechanisms including AMP production, lysis, phagocytosis, and melanization or triggers signal transduction resulting in the activation of these aforementioned immune reactions (Adapted from Kafatos.openwetware.org).

1. Non-Self Recognition through Pattern Recognition Receptors

Invertebrate innate immunity is initiated when a panel of genome-encoded, non-variant, soluble or membrane-bound pattern recognition receptors (PRRs), recognize pathogen-associated molecular patterns (PAMPs) that decorate microbial surfaces but are absent from host cells. In *A. gambiae*, bioinformatic analysis of the genome revealed the presence of several putative PRRs, which include the peptidoglycan

recognition proteins (PGRPs), the Gram-negative bacteria-binding proteins (GNBPs), the fibrinogen-related proteins (FREPs), the Immunoglobulin (Ig) domain proteins, the leucine-rich repeat proteins (LRRs), and the C-type lectin-like proteins (CTLs) (Christophides, *et al*., 2002; Waterhouse, *et al*., 2007). Although TEPs were initially classified as PRRs, subsequent functional and molecular studies suggest that they act most likely as effector molecules and hence will be discussed under effector mechanisms.

a. Peptidoglycan recognition proteins (PGRPs)

PGRPs are conserved between mammals and insects, and are specific to bacterial recognition (Dziarski, *et al*., 2003; Wang, *et al*., 2003;Kim, *et al*., 2008). In insects, these PGRPs are classified based on size into two subclasses; the long PGRPL subclass which includes transmembrane or intracellular proteins (Charroux, *et al*., 2009; Waterhouse, *et al*., 2007), and the short PGRPS subclass that are soluble. Structurally, these PGRPs have at least one C-terminal PGRP domain that is highly similar to the bacterial type 2 amidases sequence (Dziarski, 2004). In some PGRPs, the amidase enzymatic activity is lost; while in others it is retained and plays a central role in scavenging peptidoglycan leading to the dampening of immune responses (Leulier, *et al*., 2006; Girardin and Philpott, 2006).

PGRPs have been well studied in *Drosophila* where they are implicated in the activation of the Imd and Toll pathways (Filipe, *et al*., 2005; Kaneko, *et al*., 2004; Leulier, *et al*., 2003; Bischoff, *et al*., 2004). PGRPLC is, in fact, the receptor of the Imd pathway that binds directly to the PGN muropeptide of Gram-negative bacteria (Choe, *et al*., 2002; Gottar, *et al*., 2002). It is expressed in the gut, hemocyte, and fat body. Secreted PGRPLE acts also in concert with PGRPLC in activating the Imd pathway in

the midgut (Cherry and Silverman, 2006); however, intracellular PGRPLE in the hemocytes triggers the Imd independent autophagous response against *L.monocytogenes* infections (Yano, *et al*., 2008), suggesting a broader role of PGRPs in insect immunity. On the other hand, two other PGRPs, PGRPSA and PGRPSD, cooperate with GNBPs, to activate the Toll pathway in response to of Gram-positive bacterial infections (Michel, *et al*., 2001; Bischoff, *et al*., 2004). In both *Drosophila* (PGRPLC) and the silkworm *Bombyx mori*, PGRPs were also shown to activate the melanization response (Kim, *et al*., 2008). In *A. gambiae*, seven putative PGRPs have been identified; of these, PGRPLC was shown to recognize Gram-negative and Gram-positive bacteria, triggering the activation of AMP production. PGRPLC was also implicated in controlling symbiotic bacterial proliferation post blood feeding, and hence indirectly interfering with the development of malaria parasites invading the gut (Meister, *et al*., 2009).

b. β glucan recognition proteins (βGRPs)

βGRPs were initially identified in *Bombyx mori* as receptors that recognize fungal β-1,3-glucans (Ochiai and Ashida, 1988). A similar protein was later identified in *B. mori* and called GNBP because it recognizes Gram-negative bacterial infections (Lee, *et al*., 1996). GNBPs are known to recognize and bind to components of the fungal and bacterial cell walls (Kim, *et al*., 2000). βGRPs and GNBPs have an N-terminal glucan recognition domain and a C-terminal glucanase-like domain which in general lacks catalytic activity due to the loss of two glutamic acid residues (Pauchet, *et al*., 2009). βGRPs were identified and studied in several insect species namely in *B. mori* , *Drosophila*, and *A. gambiae*. In *Drosophila* for instance, GNBP1 cooperates with PGRPSA in Gram-positive bacterial recognition (Gobert, *et al*., 2003a) and thus

promotes Toll pathway activation. GNBP3, another GNBP subclass in *Drosophila*, also activates the Toll pathway after recognition of fungal cell wall constituents (Gottar, *et al.*, 2006). GNBP3 was also shown to interact with phenoloxidases and trigger melanization of fungi soon after invasion, in a Toll independent manner (Matskevich, *et al.*, 2010).

In *A. gambiae*, bioinformatic analysis of the genome identified the presence of six GNBPs. Among those, GNBPB4 has the most significant role in defense against a broad range of pathogens, including Gram-negative, Gram-positive bacteria, as well as *P. falciparum* and *P. berghei* parasites. Depleting GNBPB4 in *Anopheles* renders these mosquitoes more susceptible to the aforementioned pathogens (Warr, *et al.*, 2008).

c. <u>Fibrinogen-related proteins (FREPs)</u>

Similar to PGRPs, FREPs are another type of PRRs that exist in both vertebrates and invertebrates and contribute significantly to several immune responses. FREPs are structurally diverse and characterized by the presence of a C-terminal fibrinogen-like domain that is essential for the coagulation process in vertebrates, and that is highly linked to immunity and pathogen recognition in invertebrates. In mammals for example, ficolins mediate complement activation and phagocytosis (Fujita, 2002; Lu and Le, 1998), while tachylectins TL5A and TL5B in the *Tachypleus tridentatus* horseshoecrab are engaged in bacterial coagulation and promote clumping of human red blood cells, suggesting also a role in agglutination (Gokudan, *et al.*, 1999). FREP4, another example of fibrinogen-related proteins in the schistosome snail host *Biomphalaria glabrata*, specifically recognizes and binds to the surface of invading parasites in these invertebrates (Zhang, *et al.*, 2008). In *Anopheles*, FREPs represent the largest group of PRRs including 59 putative members as compared to 14 in *Drosophila* (Waterhouse, *et*

al., 2007). Following challenges with bacteria, fungi, and *Plasmodium*, several *Anopheles* FREPs are transcriptionally up-regulated (Dong and Dimopoulos, 2009). One particular candidate is FBN9 which interacts with the surfaces of Gram-negative and Gram-positive bacteria. Also, functional genetic studies by RNAi revealed that FBN9 has potent anti-*P. falciparum* and *P. berghei* activities (Dong, *et al*., 2006). Interestingly, the JNK pathway seems to regulate the expression of FBN9 in hemocytes in response to infection (Garver, *et al*., 2013).

d. Immunoglobulin (Ig) domain proteins

Unlike vertebrates, invertebrates lack antibodies that recognize even minor modifications in microbial antigens. Yet, around 140-150 genes with Ig domains were identified in each of the *D. melanogaster* and in *A. gambiae* genomes. (Dong, *et al*., 2006b). In *Drosophila*, the Down syndrome cell adhesion molecule (Dscam) can generate more than 18000 protein isoforms due to alternative splicing of variable exons. *Drosophila* Dscam is expressed in fat body, hemocytes, and secreted into hemolymph where it is involved in the phagocytic uptake of bacteria by hemocytes (Watson, *et al*., 2005). In *A.gambiae*, Dscam acts as a hypervariable receptor that mediates phagocytosis of *E. coli* and *S. aureus* and exhibits anti-*Plasmodium* effects (Dong, *et al*., 2006a; 2006b). Yet, the anti-parasitic mechanism of action mediated by Dscam remains unknown. *A. gambiae* Dscam undergoes pathogen specific alternative splicing following infection. Interestingly, studies in *A. gambiae* cell line Sua5B revealed that the splicing factors Caper and IRSF1, acting downstream of the Toll and Imd pathways, seem to control the repertoire of the pathogen-specific Dscam splice variants through NF-κB mediated transcription (Dong, *et al*., 2012).

e. The C-type lectins

The large C-type lectin-like domain superfamily (CTLD) includes both proteins with a carbohydrate recognition domain (CRD) which require Ca^{2+} for binding and those lacking CRDs (Zelensky and Gready, 2005). In mammals, proteins with CTLDs are classified into 17 groups; insects and most invertebrate CTLDs belong to group VII, which includes small molecular weight proteins containing one CTLD and lacking other accessory domains (Drickamer and Fadden, 2002; Zelensky and Gready, 2005).

CTLs can activate diverse effector responses that promote the clearance of microbes. In vertebrates, mannose-binding lectins (MBLs) activate the lectin complement pathway leading to microbial lysis or phagocytosis (Fujita, 2002; Schweinle, *et al.*,1989) whereas surfactant proteins A and D opsonize microbes in the respiratory tract of the infected host (Kuroki, *et al.*, 2007). In invertebrates as well, CTLs are involved in complement activation (Fujita, 2002), cellular encapsulation (Yu and Kanost, 2004) opsonization (Jomori and Natori, 1992; Wilson and Ratcliffe, 1999), nodule formation (Koizumi, *et al.*, 1999), and melanization (Yu, *et al.*,1999; Yu and Kanost, 2000; 2004). Small soluble CTLs can also act directly as effector molecules against bacteria in both vertebrates and invertebrates. In mice and humans for example, the soluble intestinal CTLs, RegIIIγ and HIP respectively, interact with and disrupt bacterial surfaces in a yet unknown manner (Cash, *et al.*,2006). Also, soluble CTLs in *Amphioxus* interact with peptidoglycan and glucans of bacterial cell walls and are directly involved in microbial killing (Yu, *et al.*, 2007).

In *A. gambiae*, bioinformatic analysis revealed 23 different members of the CTLD superfamily having different carbohydrate specificity based on their primary sequence signatures. Accordingly, these members were further classified into subgroups

(Christophides, *et al*., 2002; Waterhouse, *et al.*, 2007): mannose-binding CTLs (CTLMA subgroup), galactose-binding CTLs (CTLGA subgroup), and CTLs lacking a sugar-binding domain (CTL subgroup) (Christophides, *et al*.,2002; Drickamer, 1992). In the mosquito hemolymph, two CTLs, CTL4 and CTLMA2, form a disulfide-linked heterodimer that acts as parasite agonist; silencing either of these genes promotes *P. berghei* ookinetes melanization in S mosquitoes and blocks the development of parasites to the oocyst stage (Osta, *et al.*, 2004). More recent unpublished data revealed that these CTLs are recruited to *Plasmodium* surfaces in a manner dependent on the mosquito complement-like protein TEP1, where they are thought to be involved in the clearance or sequestration of dead parasites by midgut epithelial cells to avoid an exaggerated immune response against the parasite (Osta and Christophides, unpublished). These CTLs were also shown to be required for mosquito defense against Gram-negative, but not Gram-positive, bacterial infection (Schnitger, *et al.*, 2009).

2. CLIP-domain serine protease cascade and signal transduction

In both vertebrates and invertebrates, a broad spectrum of physiological processes including non-self recognition and complement activation, blood/hemolymph coagulation, activation of the Toll pathway during embryogenesis and fibrinolysis are tightly regulated by a diverse group of chemotropism-like serine proteases that generally function in the form of a cascade resulting in signal amplification, and in triggering downstream effector responses (figure 3).

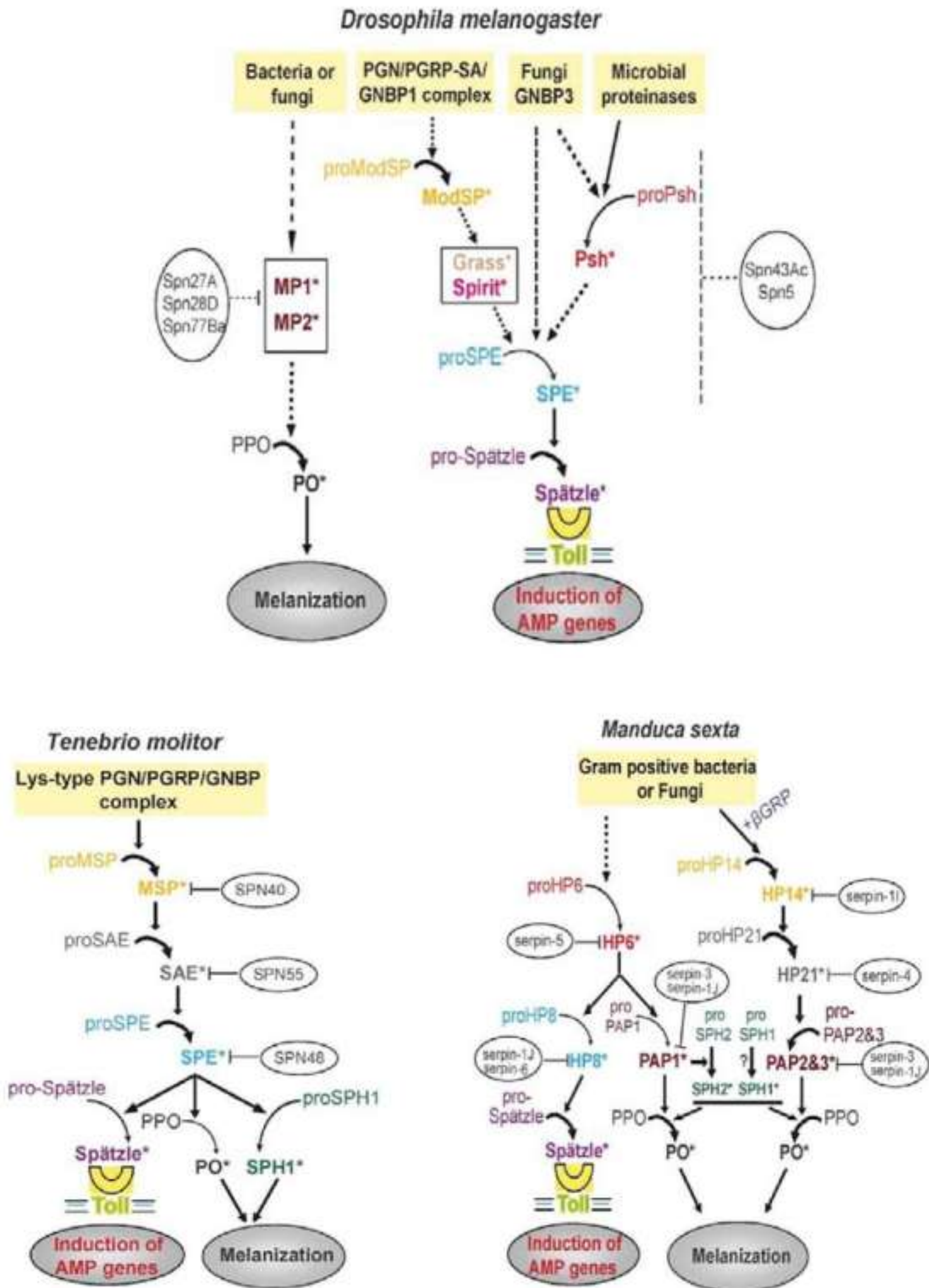


Figure 3. A proposed model of the role of protease cascades in innate immunity of three different insect species. After the binding of PRRs to microbial surfaces, an unknown mechanism promotes the auto-activation of a modular serine protease, which, in turn, triggers the activation of downstream clip domain serine proteases and protease homologs pathways. The final protease in the pathway either promotes the cleavage and activation of proPO zymogen into active PO, the key enzyme of melanization, or the

cleavage of proSpatzle into an active ligand that binds to the Toll receptor and activates AMP synthesis. Serpins tightly regulate these serine proteases to prevent any over-activation of the abovementioned immune effector mechanisms (Adapted from Noonin, *et al*., 2010).

a. The Structure and function of clip domain serine proteases

Serine proteases are key players in innate immunity; upon non-self recognition, extracellular cascades of serine proteases are activated, which relay and amplify this recognition signal either to surface receptors that activate downstream transduction pathways culminating in the expression of immune effector genes, or directly to effector molecules already present in the hemolymph. These protease cascades are mainly composed of clip-domain serine proteases (CLIPs). Structurally, CLIPs are characterized by having a disulphide-knot N-terminal domain (unique to arthropods) and a C-terminal trypsin-like domain with a His-Asp-Ser triad in the catalytic site (Muta, *et al*., 1993). A subfamily of CLIPs, known as serine protease homologs (SPHs), is considered enzymatically inactive because of a serine to glycine substitution in their catalytic site. Catalytic CLIPs and SPHs are sometimes called easter-type and masquerade-type serine proteases respectively, due to their high structural resemblance and sequence similarities with the two *Drosophila* CLIPs easter and masquerade involved in embryonic development (Chasan and Anderson, 1989; Murugasu-Oei, *et al*., 1995). In CLIPs, the clip domain which is usually composed of around 30-60 amino acid residues containing three disulfide bonds is attached to the serine protease (SP) domain by means of a linker region of variable length ranging between 23-92 residues. A cysteine residue in the linker region forms a disulfide bond with another residue in the SP domain. A recent study has further classified catalytic CLIPs into two groups; one group that contains penultimate proteases devoid of an additional loop (75-loop) in

the proximity of the activation site localized on a protruding site in the catalytic domain, and another 75-containing loop with terminal proteases (Kellenberger, *et al.*, 2011). Serine proteases are secreted to the hemolymph as zymogens; which require proteolytic cleavage in the linker region to become active (Kim, *et al.*, 2002; Piao, *et al.*, 2005). Nevertheless, the N-terminal fragment containing the clip domain remains covalently attached to the C-terminal SP domain by a disulfide bond after cleavage (figure 4) (Jiang and Kanost, 2000). Unlike the serine protease domain that is known for its role in proteolysis and in mediating signal amplification, the clip domain does not seem to have yet a defined role. It is believed that clip domains might mediate protein-protein interactions with PRRs, with other clip domain serine proteases in the cascade, with phenoloxidases, or with cofactors such as SPHs ensuring specificity and tight spatial and temporal regulation of these cascades (Jiang and Kanost, 2000; Piao, *et al.*, 2005; Huang, *et al.*, 2007).

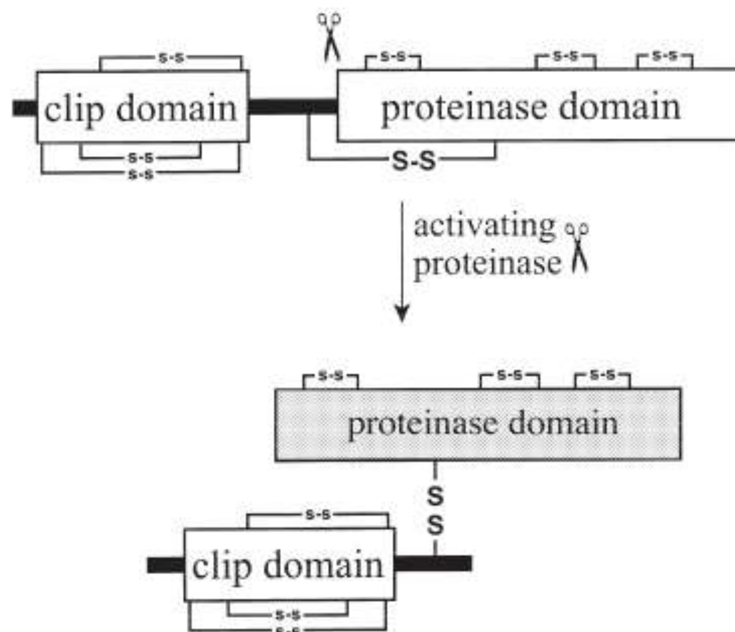


Figure 4. Mechanism of CLIPs activation. Proteolytic cleavage at the site between the clip domain and the serine proteinase domain converts the CLIP from a zymogen into

an active form. A disulfide bond links the two cut fragments together (Adapted from Jiang and Kanost, 2000).

b. The regulation of serine proteases by serpins

Serine protease inhibitors, also known as Serpins (SRPNs), belong to a protein superfamily that is involved in tightly regulating (mainly inhibiting) proteases, mostly those belonging to the serine class (Reviewed in Gulley, *et al.*, 2013). The exposed reactive center Loop (RCL) in Serpins promotes its binding to the protease at their active sites, in an enzyme-substrate manner, resulting in the formation of covalently and irreversibly bound serpin-protease complexes. Serpins are important in controlling the extent of amplification of immune responses to avoid exaggerated immunity that could impose a fitness cost upon the host (Reichhart, 2005; Reichhart, *et al.*, 2011). For instance, serpin 27A mutants of *D. melanogaster* exhibit spontaneous melanization in tissues of both larvae and adult flies (Ligoxygakis, *et al.*, 2002b). In *A.gambiae*, SRPN2 kd also triggered the appearance of spontaneous pseudotumors in tissues, associated with a reduction in the mosquito lifespan (Michel, *et al.*, 2005). The absence of SRPN2 in these mosquitoes significantly increased *P. berghei* ookinete lysis and melanization resulting in a significant increase in the oocyst numbers (Michel, *et al.*, 2005). However, a similar phenotype was not seen against both *P. falciparum* in autochthonous *A.gambiae* strains (Michel, *et al.*, 2006) and *P. gallinaceum* in *Ae. aegypti* (Zou, *et al.*, 2010). SRPN6, was also shown to be a functional serine proteinase inhibitor whose endogenous target proteinase(s) are yet to be identified (An and Michel, unpublished, Osta MA personal communication). In some mosquito species, like *A. stephensi*, SRPN6 has distict roles and is considered a biomarker for *Plasmodium* invasion, whereby silencing SRPN6 resulted in a significant increase in the number of

developing *P. berghei* oocysts (Abraham, *et al*., 2005; Pinto, *et al*., 2008). In *A. gambiae* however, SRPN6 kd doesn't seem to exert an effect on the number of developing parasites, but is more involved in the process of parasite lysis (Reviewed in Gulley, *et al*., 2013) (figure 5).

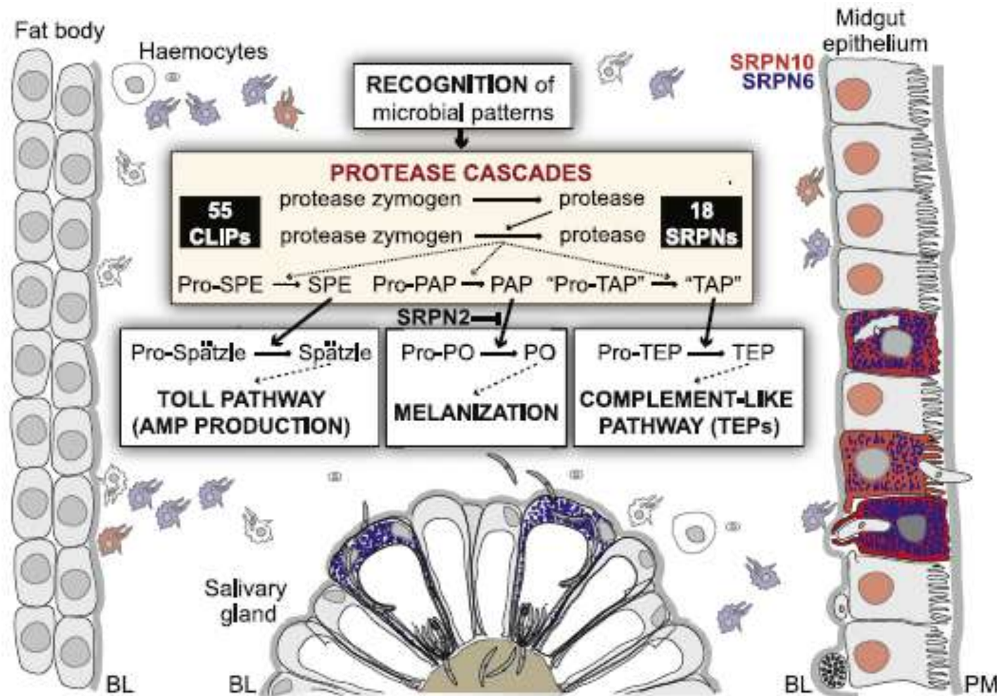


Figure 5. The role of Serpins in regulating mosquito immune responses.
Midgut epithelia, salivary glands, hemocytes, and the fat body are the major tissues that contribute to immune responses against human pathogens. In the hemolymph, and upon pathogen recognition, the proteinase cascades are triggered, promoting the activation of different downstream immune reactions: (a) antimicrobial peptide production through the activation of the Toll pathway; (b) melanization through phenoloxidases activation; (c) thioester proteins activation through the activation of the complement pathway. Although serpins are involved in diverse physiological processes; the majority of the characterized serpins are mainly involved in regulating these immune responses (Adapted from Gulley, *et al*., 2013).

c. The role of CLIPs in insect innate immunity

i. Activation of prophenoloxidase

Phenoloxidase (PO) is a key enzyme of the melanization immune response in insects. Terminal CLIPs (also known as PPAE) are involved in converting PPO to active PO by cleaving a 5-10 kd fragment from the N-terminal domain (Ashida and

Brey 1997). The fact that quinone intermediates produced during the melanotic immune response are highly toxic, the tight spatial and temporal regulation of PPO-activation becomes imperative. Most biochemical work on PPO activation by PPAE was done in lepidopterans and coleopterans due to their relatively large size that allows the extraction of sufficient amounts of hemolymph for protein purification and *in vitro* reconstitution assays. Studies in the large beetle *H.diomphalia* for example showed that the clip-domain serine protease homologue PPAF-II undergoes a conformational change following cleavage to allow its binding to PO, facilitating PO cleavage into its final catalytic form (Piao, *et al*., 2005). In *H.diomphalia* also, both catalytic CLIPs and SPHs are needed for PPO activation (Kim, *et al*., 2002; Kwon, *et al*., 2000; Lee, *et al*., 1998). In this model, PPAF-I acts as the terminal CLIP in the cascade, promoting the direct cleavage of proPO (79kda) into its active form PO (76kda). Yet, PO alone is still considered inactive, and requires proper complexing with an activated PPAF-II produced by cleavage of pro-PPAF-II by the catalytic PPAF-III, to exhibit full PO activity (Piao, *et al*., 2005). In the tobacco hornworm *Manduca sexta*, three different CLIPs called prophenoloxidase-activating proteins (PAPs) are involved in activating PO. PAP-1 contains one CLIP domain, while PAP-2 and PAP-3 each contain two CLIP domains (Jiang, *et al*., 1998; Jiang, *et al*., 2003a; Jiang, *et al*., 2003b). PAP-1, PAP-2, and PAP-3 cannot activate PO by themselves and require two SPHs (SPH-1 and SPH-2) to efficiently cleave and activate PPO. SPH-1 and SPH-2 are proposed to strengthen the interactions between PAP-1 and PPO, thus promoting a proper spatial orientation and cleavage of PPO (Yu, *et al*., 2003). In this study as well, SPH-1 was also shown to exist in the same complex with PPO, PAP-1, and immunulectin-2, a lipopolysaccharide-binding CTL (Yu, *et al*., 1999; Yu and Kanost, 2000), suggesting a possible role for these SPHs in PO localization to microbial surfaces by interacting with PRRs.

Additional biochemical studies in *M. sexta* highlighted the complexity of protease cascades acting upstream of PPO activation. HP14, a non-clip pattern recognition modular serine protease, binds to PAMPs on microbial surfaces leading to its autoactivation (Ji, *et al.*, 2004) and directs the cleavage of proHP21 (a hemolymph CLIP) into active HP21 (Wang and Jiang, 2004). Once activated, HP21 then cleaves PAP2 and PAP3 which generates active PO in the presence of SPH-1 and SPH-2 (Ji, *et al.*, 2004; Wang and Jiang, 2007). Though well established in lepidopteran and coleopteran, the role of the CLIP cascade in activating PPO remains poorly understood in *Drosophila*. A recent study in serpin spn27A mutant flies exhibiting a spontaneous melanotic response (De Gregorio, *et al.*, 2002) failed to show a direct role for MP1 and MP2, two CLIP proteases, in PO cleavage (Jang, *et al.*, 2008). Although the *Drosophila* PPAEs remain unknown; PPO activation is tightly controlled in these flies and exaggerated melanization is prevented by a strong regulation exerted by an induced expression of the spn27A target protease through the Toll pathway (Leclerc, *et al.*, 2006; Ligoxygakis, *et al.*, 2002b).

ii. Activation of the Toll pathway

In *Drosophila*, the transmembrane receptor Toll was first identified to be involved in the development of the flies' dorso-ventral axis. In this pathway, two upstream CLIPs (easter and snake) were shown to be involved in the proteolytic cleavage of inactive prospatzle into active spatzle which binds to the Toll receptor and activates the pathway resulting in a proper arrangement of dorso-ventral polarity during development (DeLotto and DeLotto, 1998). Later, genetic studies revealed an important contribution of the Toll signaling pathway to *Drosophila* innate immunity as well (Lemaitre, *et al.*, 1996). Though Toll-like receptors (TLRs) also exist in mammals

namely in humans (Medzhitov, *et al.*, 1997) and in mice (Poltorak, *et al.*, 1998a; 1998b); however, their mode of action differs from those of *Drosophila*. Mammalian TLRs directly recognize and bind to PAMPs on microbial surfaces (Yang, *et al.*, 1998), unlike in *Drosophila* where recognition occurs by upstream PRRs belonging to PGRPs and GNBPs families (Gobert, *et al.*, 2003b; Gottar, *et al.*, 2006; Kim, *et al.*, 2000; Levashina, *et al.*, 1999a), leading to the activation of specific proteolytic cascades, promoting spatzle cleavage and Toll activation (Jang, *et al.*, 2008).

Several CLIPs were shown to be key players upstream of spatzle cleavage. In Drosophila for instance, *in vitro* studies revealed a direct role of Spz Processing Enzyme (SPE) CLIP in cleaving prospatzle in response to fungal and Gram-positive bacterial infections (Jang, *et al.*, 2006; Jang, *et al.*, 2008). RNAi functional genetic screens identified grass and spirit, two additional *Drosophila* CLIPs, as being important members of the Toll-activating cascades (Kambris, *et al.*, 2006). In the mealworm *Tenebrio molitor*, a similar Spatzle processing CLIP enzyme (TmSPE) was also shown to activate the Toll signaling pathway (Kim, *et al.*, 2008). As previously mentioned, these CLIPs are always under tight regulation to prevent exaggerated immunity. Fungal infected spn43Ac mutant flies for example, exhibit constitutive Toll activation and continuous expression of the AMP drosomycin (Levashina, *et al.*, 1999a) due to a non-stop activation of a CLIP-SP target protease known as persphone (psh), promoting this unconstrained immune activation (Ligoxygakis, *et al.*, 2002a).

iii. CLIPs in *A. gambiae* innate immunity

In the malaria vector *A. gambiae,* several CLIPs and SPHs have been involved in immune responses directed against *Plasmodium* parasites (Povelones, *et al.*, 2013;Volz, *et al.*, 2006; Volz, *et al.*, 2005), bacteria (Volz, *et al.*, 2005; Dong, *et al.*,

2006b; Dong, *et al*., 2009), and fungi (Yassine, *et al*., 2012). Phylogenic analysis classified the 54 identified mosquito CLIPs into five subfamilies, A, B, C, D and E, based on their sequence-specific signature (Waterhouse, *et al*., 2007). Functionally, these CLIPs may be grouped into two categories, catalytic CLIPs (B, C, D) and non-catalytic CLIPs (A and E) or serine protease homologs (SPHs). Clip-SPHs have been mainly implicated in triggering the melanization immune response in several insect species (Kim, *et al*., 2002; Schnitger, *et al*., 2007; Yu, *et al*., 2003); however their mechanisms of action and their broader role in immunity remain poorly investigated. In *A. gambiae*, functional in vivo genetic analysis of selected CLIPs (10 CLIPAs and 11 CLIPBs) using RNA interference identified genes with opposing ookinete melanization phenotypes. Noticeably, the genetic background of the mosquito highly influenced the functional role of some of these CLIPs (figure 6).

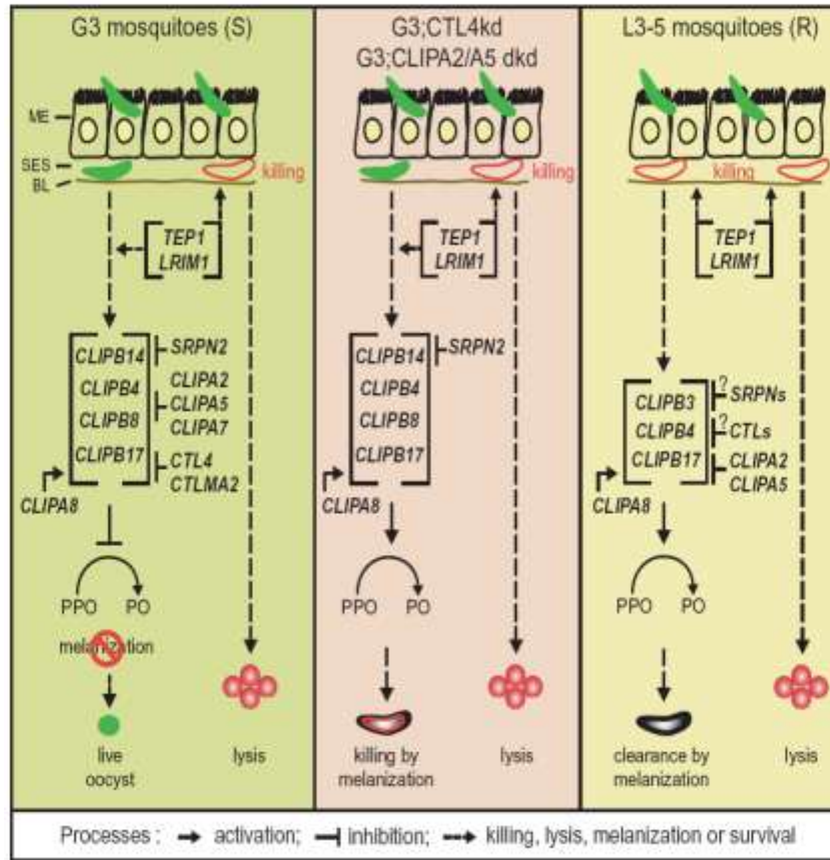


Figure 6. Melanization in different *A.gambiae* genetic backgrounds. In susceptible (S) mosquito (left panel), around 20% of the invading parasites escape TEP1 mediated killing in the sub-epithelial space (SES). The tight regulation of the melanotic cascade in these mosquito backgrounds exerted by serpin, CLIPAs, and CTLs prevents ookinete melanization. In *CTL4*kd or *CLIPA2/CLIPA5*dkd mosquitoes (middle lane), TEP1-dependent melanization is activated and results in ookinete killing. In refractory (R) mosquito strains (right panel), melanization is inherently active, whereby all invading ookinetes are killed in TEP1-dependent manner and cleared by melanization. In this melanotic pathway, CLIPA8 and some CLIPBs positively regulate the melanization cascade, while CLIPA2 and CLIPA5 exhibit inhibitory roles. (Adapted from Volz, *et al.*,2006).

Among non-catalytic CLIPAs, only CLIPA8 acts a positive regulator of ookinete melanization in both R strains and *CTL4*kd S strains. *CLIPA8kd* completely blocked *P. berghei* melanization in these mosquito genotypes. Other CLIPAs, namely CLIPA2, CLIPA5, and CLIPA7 were shown to act as negative regulators of the melanization response. In *CLIPA2/CLIPA5*dkd phenotypes, 88.3% of the invading

parasites are melanized in susceptible G3 mosquitoes. In R mosquitoes however, simultaneous silencing of *CLIPA2* and *CLIPA5* enhances the number of melanized parasites. On the other hand, most catalytic CLIPBs like CLIPB17 and CLIPB4 (in different genetic backgrounds), CLIPB3 (in R strains), and CLIPB8 (in *CTL4*kd S strains) are positive regulators of *P. berghei* melanization (Volz, *et al*., 2006). Reverse genetic and biochemical analysis identified CLIPB9 as the first *A.gambiae* CLIP that is directly involved in PPO cleavage. CLIPB9 is negatively regulated by SRPN2 and the double knock down of *SRPN2* and *CLIPB9* reversed the melanization phenotype exhibited by SRPN2kd (An, *et al*., 2011).

Apart from the role of CLIPs in regulating parasite melanization, they are also involved in melanization-independent parasite killing. Silencing either *CLIPB14*, *CLIPB15* (Volz, *et al*., 2005), or *SPCLIP1* (Dong, *et al*., 2006a) in non-melanization S strains lead to a significant increase in the numbers of invading parasites in these mosquito backgrounds. In R mosquitoes, CLIPB14 and CLIPB15 work in a synergistic manner; the simultaneous silencing of these two genes enhances the number of melanized parasites. Interestingly, a recent study identified a functional link between SPCLIP1 and the complement-like protein TEP1. SPCLIP1 was shown to co-localize with TEP1 on ookinete and bacterial surfaces whereby it seems to facilitate the conversion of TEP1-F to $TEP1_{cut}$, suggesting the existence of a complement convertase-like activity in the mosquito analogous to that operating in the mammalian immune system (Povelones, *et al*., 2013). In addition to the role of SPCLIP1 in regulating TEP1, other SPHs like CLIPA2 (partly characterized in the context of this thesis work) also seem to be involved in this regulatory process. CLIPA2 is recruited to pathogenic surfaces in a TEP1-dependent manner and acts as a negative regulator of TEP1 consumption during systemic bacterial infections (Yassine, *et al*., 2014). CLIPA9,

another SPH, is also involved in immune defense against both *P.berghei* and *P.falciparum* (Dong, *et al*., 2006a); *CLIPA9* kd enhances *P.falciparum* lysis in septic but not aseptic mosquitoes, suggesting that CLIPA9 might be mediating its effect indirectly through the control of the microbiota (Dong, *et al*., 2009).

Besides their role in parasite immunity; some of the aforementioned CLIPs are also engaged in anti-bacterial and anti-fungal immune responses. The absence of CLIPA8 for example abolishes bacterial melanization in the hemocoel of *A.gambiae* mosquitoes (Schnitger, *et al*., 2007) and prevents melanin formation on *B. bassiana* hyphae in infected mosquitoes (Yassine, *et al*., 2012). Unlike CLIPA8 however, whose absence doesn't affect mosquitoes' survival to bacterial infections (Schnitger, *et al*., 2007), CLIPB14, CLIPB15 and SPCLIP1 are required for mosquito survival after bacterial challenge (Volz, *et al*., 2005; Dong, *et al*., 2006a). Apart from the well established functions of several CLIPs especially SPHs, their mechanism of action and how do they contribute to anti-microbial defense remain poorly understood.

3. Immune signaling pathways in mosquito immunity

In *Drosophila*, several studies revealed the presence of two main immune signaling pathways; Toll and Immune deficiency (Imd). The Toll and Imd pathways regulate AMP gene expression in response to Gram-positive and Gram-negative infections, respectively. Moreover, the Toll pathway is also activated following fungal infections whereby it triggers the expression of the anti-fungal AMP genes *Drosomycin* and *Metchnikowin* (Reviewed in Lemaitre and Hoffman, 2007). In flies, the Toll pathway also regulates the arrangement of dorso-ventral polarity during embryonic development (DeLotto and DeLotto, 1998). The Toll pathway activates the NF-κB-like transcription factors DIF (Dorsal-related immunity factor) and Dorsal in response to

infection and during embryonic development, respectively; whereas the Imd pathway activates NF-κB-like transcription factor Relish (Anderson, *et al.*, 1985; Doyle, *et al.*, 1989; Nusslein-Volhard, *et al.*, 1980).

Comparative genomic analysis showed that several genes downstream of the Toll and Imd pathways in *Drosophila* are conserved in *A. gambiae*, with few exceptions. Surprisingly, an orthologous for *Drosophila* NF-κB transcription factor DIF is absent from mosquitoes (Christophides, *et al.*, 2002). Rel1 and Rel2 are the only two mosquito NF-κB transcription factors orthologous to *Drosophila* Dorsal and Relish, respectively (Christophides, *et al.*, 2002; Waterhouse, *et al.*, 2007). Similar to its DIF analogue in *Drosophila*, mosquito Rel1 is also regulated by the Toll pathway. Both DIF and Rel1 are controlled by the negative regulator Cactus, which sequesters them in the cytoplasm and masks their nuclear localization signal. In *Drosophila*, upon Toll activation however, Cactus gets phosphorylated and subsequently degraded, promoting DIF nuclear translocation, eventually activating target genes (figure 7) (Belvin and Anderson, 1996; Belvin, *et al.*, 1995). In *A. gambiae*, Rel1 pathway is involved in immune defense against *Plasmodium* parasites; silencing *cactus* promotes the over activation of the pathway resulting in complete refractoriness to *P.berghei* (Frolet, *et al.*, 2006; Garver, *et al.*, 2009), and in a significant reduction in the number of live *P.falciparum* oocysts (Garver, *et al.*, 2009). Noticebaly, the absence of cactus was assosiated with a significant elevation in the basal levels of a number of anti-parasitic genes such as *TEP1*, *LRIM1*, *APL1C*, among others, pointing out to an important contribution of the Rel1 pathway to the mosquito's basal immunity which exhibit strong negative impact on malaria parasites invading the gut epithelium (Frolet, *et al.*, 2006; Riehle, *et al.*, 2008). The Toll/Rel1 pathway was also implicated in anti-viral (Xi, *et al.*,

2008; Ramirez and Dimopoulos, 2010) and anti-fungal immunity in the dengue vector *A.aegypti* (Shin, *et al*., 2005; 2006).

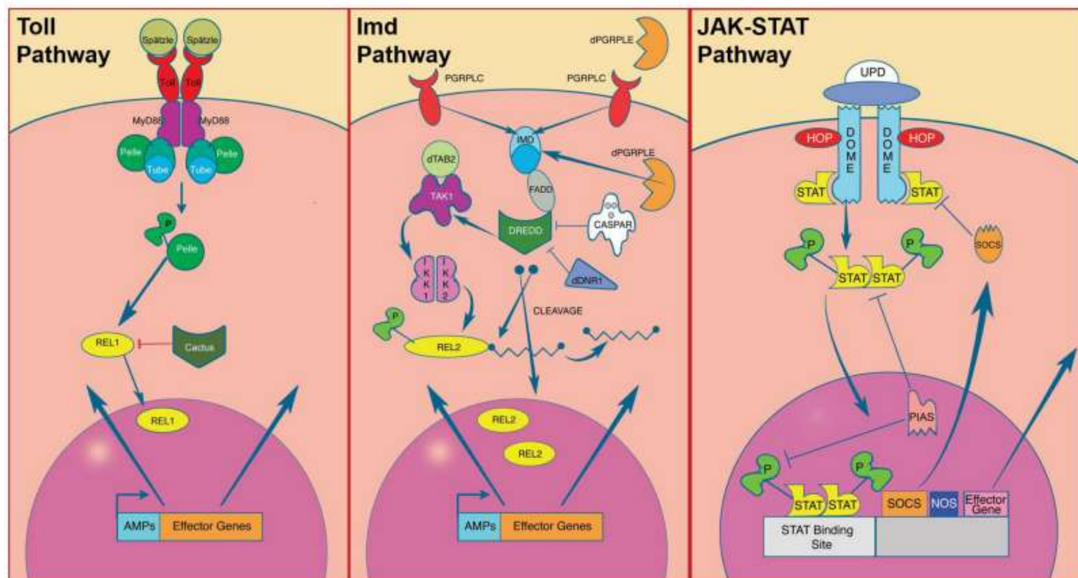


Figure 7. The role of the Toll, Imd, and JAK-STAT signaling pathways in immune defense. The recognition of bacteria or *Plasmodium* promotes the binding of the Spätzle ligand to the Toll transmembrane receptor, activating the Toll pathway. A series of molecular events then take place, resulting in Rel1 nuclear translocation and, in turn, transcriptional up-regulation of several immune genes involved in microbial killing (first lane). Among different immune pathways, the Imd pathway seems to be the most efficient in defense against *P. falciparum*, the human malaria parasite. This pathway is activated upon bacterial or *Plasmodium* recognition by the transmembrane receptor PGRP-LC, leading to a downstream activation of a signaling cascade, culminating in the cleavage of Rel2-F, and in the translocation of the Rel2-S into the nucleus, promoting transcriptional translocation of different immunity genes involved in microbial defense (second lane). Upon Parasite, bacterial or viral recognition, the cytokine ligand Unpaired (UPD) binds to the transmembrane receptor DOME, promoting the activation of the JAK-STAT pathway, resulting in the translocation of STAT to the nucleus, and thus the transcriptional activation of several genes involved in immunity (third lane). (Adapted from Clayton, *et al*., 2014).

In *A.gambiae,* alternative splicing of Rel2 produces two different isoforms: a full length (Rel2-F) mainly involved in defense against invading *P.berghei* parasites and Gram-positive bacterial (*Staphylococcus aureus*) infection, and a short length (Rel2-S) implicated mainly in immunity against Gram-negative bacteria (*Escherichia coli*)

(Meister, *et al.,* 2005). Subsequent studies revealed that the Rel2 pathway contributes significantly to anti- *P. falciparum* immunity. Silencing *Rel2* dramatically increases the number of developing *P. falciparum* oocysts in *A. gambiae* mosquitoes (Garver, *et al.*, 2009; Dong, *et al*., 2006; Mitri, *et al*., 2009). Constitutive activation of the Rel2 pathway by silencing its negative regulator *caspar* resulted in near refractoriness of three malaria vectors: *A. gambiae*, *A. stephensi*, and *A. albimanus* to *P. falciparum* (Garver, *et al.*, 2009). Along these lines, transgenic mosquitoes over-expressing the active form of Rel2 (Rel2-S) using blood meal-inducible promoters exhibited potent anti- *P. falciparum* activities by enhancing the expression of a combination of effector molecules including TEP1, APL1C, and LRRD7 (Dong, *et al*., 2011). These mosquitoes also exhibited increased resistance to gut bacteria and to systemic infections with *E. coli* and *S. aureus*, suggesting that the Imd/ Rel2 pathway plays non-redundant roles with Toll/ Rel1 in anti-bacterial defense. *A. aegypti* transgenic mosquitoes over expressing Rel2 displayed also increased resistance to Gram-positive and Gram-negative bacteria as well as to *P. gallinaceum* further highlighting the importance of this pathway in mosquito immunity (Antonova, *et al*., 2009). As with Rel1, Rel2 was also shown to exhibit an anti-parasitic response by controlling the basal expression levels of several anti-parasitic target genes, including *TEP1* and *LRIM1*. Noticeably, this Rel2 mediated response is thought to be *Plasmodium* species-specific (Meister, *et al.*, 2005; Graver, *et al*., 2009).

Despite playing key roles in mammalian innate and adaptive immunity, little is known about the role of the JAK-STAT pathway in insect immunity. In mosquitoes, the JAK-STAT pathway was also shown to be involved in immune defense against both *P.berghei* and *P.falciparum*. Interestingly, the STAT pathway induces anti- *Plasmodium* immunity against later oocyst stages of the parasite (Gupta, *et al.*, 2009). Two STAT

proteins exist in *A. gambiae*: *Ag*STAT-A and *Ag*STAT-B. The latter regulates *Ag*STAT-A and translocates to the nucleus of fat body cells upon bacterial infection promoting the activation of several anti-bacterial target genes (Barillas-Mury, *et al*., 1999). *Ag*STAT-A, in turn, has both anti-bacterial and anti-parasitic roles as it up-regulates the transcript levels of Nitric Oxide Synthase (NOS) leading to increased levels of reactive Nitric Oxide (NO). Besides, *Ag*STAT-A plays a role in controlling the transient transcriptional up-regulation of the anti-*Plasmodium* factor TEP1 during the post-invasion phase of the midgut, possibly serving TEP1 replenishment (Gupta, *et al*., 2009). The JAK-STAT pathway has also been implicated in defense against dengue virus in *A. aegypti* (Souza-Neto, *et al*., 2009) and in *D. melanogaster* (Dostert, *et al*., 2005).

4. Immune effector mechanisms in mosquitoes

a. <u>Synthesis of anti-microbial peptides (AMPs)</u>

The production of AMPs in response to microbial infections is a common humoral immune response in all insects. In dipterans, infection stimulates fat body (systemically) and/or barrier epithelial (locally) to produce and release large amounts of AMPs into the hemolymph (Bulet and Stocklin, 2005). AMPs are in general small cationic peptides (12-50 amino acid long) that interact with the negatively-charged cellular membrane of their target pathogen, promoting its disruption (Hoffman, 2003). However, the mode of action of AMPs is not restricted to disrupting cell wall integrity, as some AMPs alter several physiological processes within the cell (Reviewed in Nguyn, *et al*., 2011). AMPs in general have a broad spectrum of activity against bacteria and fungi and in some cases parasites and enveloped viruses (Cruz, *et al*., 2014). AMPs are broadly classified into three major classes; peptides rich in cysteine

disulfide linkages, linear peptides forming α-helices, and peptides rich in proline and/or glycine (Bulet and Stocklin, 2005). These three classes are also classified into families based on specific sequence signatures. In *Drosophila* for instance, seven AMP families have been identified: Drosocin, Attacin, Diptericin (DPT), Cecropin (CEC) (categorized as anti-Gram-negative peptides), Defensins (DEF) (anti-Gram-positive peptides), Metchnikowin and Drosomycin (anti-fungal peptides) (Hetru, *et al.*, 2003). The production of these *Drosophila* AMPs is tightly regulated by the Toll and Imd pathways as previously discussed. In *A. gambiae*, only four distinct AMP families have been identified: Cecropins (four genes), Defensins (five genes), Diptericin (one gene), and Gambicin (one gene) (Christophides, *et al.*, 2002; Waterhouse, *et al.*, 2007). This apparent scarcity of mosquito AMPs when compared to *Drosophila* is possibly due to the distinct natures of their microenvironments. Functionally, *Anopheles* AMPs are active against a broad range of microbes: CEC1 (Kim, *et al.*, 2004; Vizioli, *et al.*, 2000) and GAM (Dong, *et al.*, 2006a; Vizioli, *et al.*, 2001a) have anti-bacterial (both Gram-negative and Gram-positive) and anti-*Plasmodium* effects. CEC1 is also active against yeast. On the other hand, DEF1 is only active against some filamentous fungi and Gram-positive bacteria (Vizioli, *et al.*, 2001b).

b. <u>Phagocytosis</u>

Phagocytosis is an evolutionary conserved cellular immune response in both vertebrates and invertebrates where invading pathogens and apoptotic bodies are recognized, engulfed, and destroyed by phagocytes. In mosquitoes, three types of hemocytes are known: granulocytes, oenocytoids and prohemocytes (Castillo, *et al.*, 2006). Granulocytes constitute approximately 80% of circulating hemocytes and are highly phagocytic (Hillyer, *et al.*, 2003a; 2003b). Oenocytoids are less than 10% and are

the major producers of PPO (Castillo, *et al*., 2006), while prohemocytes are less than 10% of circulatory hemocytes and were also shown to exhibit phagocytic activity (King and Hillyer, 2013). It is estimated that adult mosquitoes contain between 500-4000 circulating hemocytes (Reviewed in Hillyer and Strand, 2014). Approximately, 75% of hemocytes in adults are circulating while 25% are sessile (King and Hillyer, 2013). Intra vital imaging techniques identified a new immune tissue composed of sessile hemocytes flanking the heart. These were called periostial hemocytes (King and Hillyer, 2012). Infection induces circulating hemocytes to migrate to periostial regions where they form aggregates with periostial hemocytes already present there. These hemocytes are highly phagocytic and are positioned strategically to rapidly eliminate microbes as they flow within the hemolymph.

A semiquantitative functional genetic screen of 71 candidates in adult *A. gambiae* mosquitoes identified 26 distinct genes involved in phagocytosis and revealed two main distinct pathways that regulate the uptake of Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria; CED5 and CED6 (intracellular homologs of *Caenorhabditis elegans*)-dependent pathways (Moita, *et al.,* 2005). Although very few phagocytic receptors or opsonins have been identified in mosquitoes so far; several key players in the CED5 and CED6 pathways has been recognized. The CED5 pathway includes, for example, the transmembrane β-intergin (BINT2), the intracellular CED2, and the soluble TEP4. The CED6 pathway, in turn, consists of the transmembrane low-density lipoprotein receptor-related protein (LRP), and the soluble TEP1, TEP3 and LRIM1 proteins (Moita, *et al.,* 2005).

Several members of the Nimrod superfamily in *Drosophila* are also involved in the phagocytosis of bacteria and apoptotic bodies (Somogyi, *et al.,* 2008; Kuraishi, *et al.,* 2009; Kocks, *et al.,* 2005); particularly Eater which is expressed in hemocytes and

is involved in phagocytosis of Gram-negative and Gram-positive bacteria (Kocks, *et al.,* 2005). In *A. gambiae*, the Nimrod member AgNimB2 was shown to mediate the phagocytosis of *S. aureus*, but not *E .coli*, via an indirect pathway that doesn't require direct binding to bacterial surfaces (Midega, *et al.,* 2013). The contribution of the two other identified members of the *Anopheles* Nimrod superfamily, draper and eater, to the phagocytic process is not clear yet, as these genes may either play redundant roles or are more engaged in mediating other immune processes, such as the up-regulation of nitric oxide synthase mRNA levels (by eater) that enhances the mosquito's ability to kill *E. coli* in the hemocoel (Estevez-Lao and Hillyer, 2014). Interestingly, a recent study has shown a role for *A. gambiae* cytoplasmic actin in mediating phagocytosis, whereby secreted Actin5c interacts with the extracellular MD2-like immune factor AgMDL1 and binds to bacterial surfaces resulting in their phagocytosis and direct killing. Actin5c was secreted to the hemolymph following bacterial systemic infections where it binds to bacteria, facilitating their up-take by granulocytes. The binding of Actin5c to bacteria was enhanced in the presence of AgMDL1. Actin5c acts also as a *Plasmodium* antagonist limiting the survival of ookinetes stage parasites in a yet unidentified manner (Simoes and Dimopoulos, 2015).

c. Complement-mediated killing

TEP1 is one of the well characterized soluble immune factors in *Anopheles*. It is a member of the thioester-containing protein family, and is a complement-like protein that structurally resembling C3 in vertebrates, but lacks the C345C and anaphylatoxin domains (Baxter, *et al.,* 2007). In mammals, several pathways converge on the cleavage of C3 into its opsonin form C3b, including the classical pathway (mediated by IgM and IgG), the lectin pathway (mediated by mannose binding lectins and ficolins), and the

alternative pathway (spontaneous hydrolysis of C3) (Reviewed in Lambris, *et al.,* 2010). Proteolytic cleavage of C3 exposes its thioester bond that attacks and covalently binds to the nucleophilic groups in its vicinity on the surfaces of microbes, triggering phagocytosis or the assembly of a membrane attack complex (MAC) that promotes pathogenic lysis (figure 8) (Blandin, *et al.,* 2008). Further, an inflammatory immune response is activated by the small C3 fragment (C3a), that also serve as a chemotactic factor guiding phagocytes to the infection site (Ricklin, *et al.,* 2010). Similar to C3, TEP1 is also cleaved and has been shown to be the hallmark of mosquito immunity.

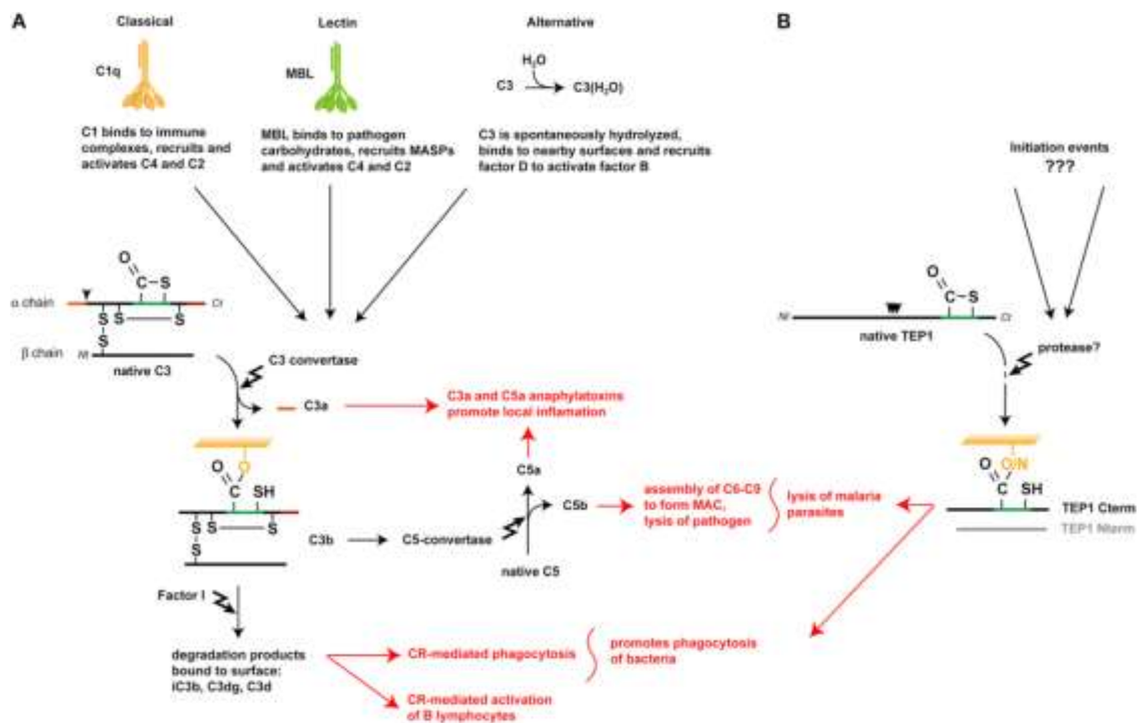


Figure 8. A comparison between the complement system in mammals and the complement-like system in mosquitoes. (a) A schematic diagram of the complement system in mammals. The activation of the complement factor C3 which is intracellularly processed and released into the blood stream as a mature molecule made of disulfide linked α and β chains happens through the classical, lectin, and alternative pathways. All three pathways converge to one point where they promote the proteolytic cleavage of C3 by C3 convertases, and the activation of the same group of effector mechanisms. The major conformational change that happens upon cleaving the C3a fragment or anaphylatoxin (represented in red) promotes the exposure of the thioester group that covalently attaches to pathogenic surfaces. (b) A scheme representing the proposed complement-like system model in *A. gambiae*. Unlike C3, the complement-like protein, TEP1, is released into the mosquito hemolymph as one single-chained molecule, which

then undergoes further processing into a two-chained non-covalently attached molecule (TEP1-N and TEP1-C). Although the molecular events underlying the proteolytic activation of TEP1 remain poorly understood; TEP1 binding to microbial surfaces also requires the activity of a functional thioester and activate similar immune effector mechanisms as those in C3, namely phagocytosis, and lysis of microbial cells. (Adapted from Blandin, *et al.,*2008).

TEP1 binds to the surface of *P.berghei* ookinetes leading to their killing through an unknown mechanism that may involve lysis (Blandin, *et al.,* 2004). It was also shown to act against *P. falciparum* (Dong, *et al.,* 2006b); however, its binding to the human malaria parasite has not been determined. Additionally, the clearance of Gram-positive and Gram-negative bacteria from hemolymph by phagocytosis requires TEP1, which acts as an opsonin that covalently binds to bacterial surfaces in a thioester dependent manner (Levashina, *et al.,* 2001; Moita, *et al.,* 2005). TEP1 plays a significant role in anti-fungal immunity as well, where it interfers with the invading *Beauvaria bassiana* in *A. gambiae* and retards its growth (Yassine, *et al.,* 2012) (figure 9).

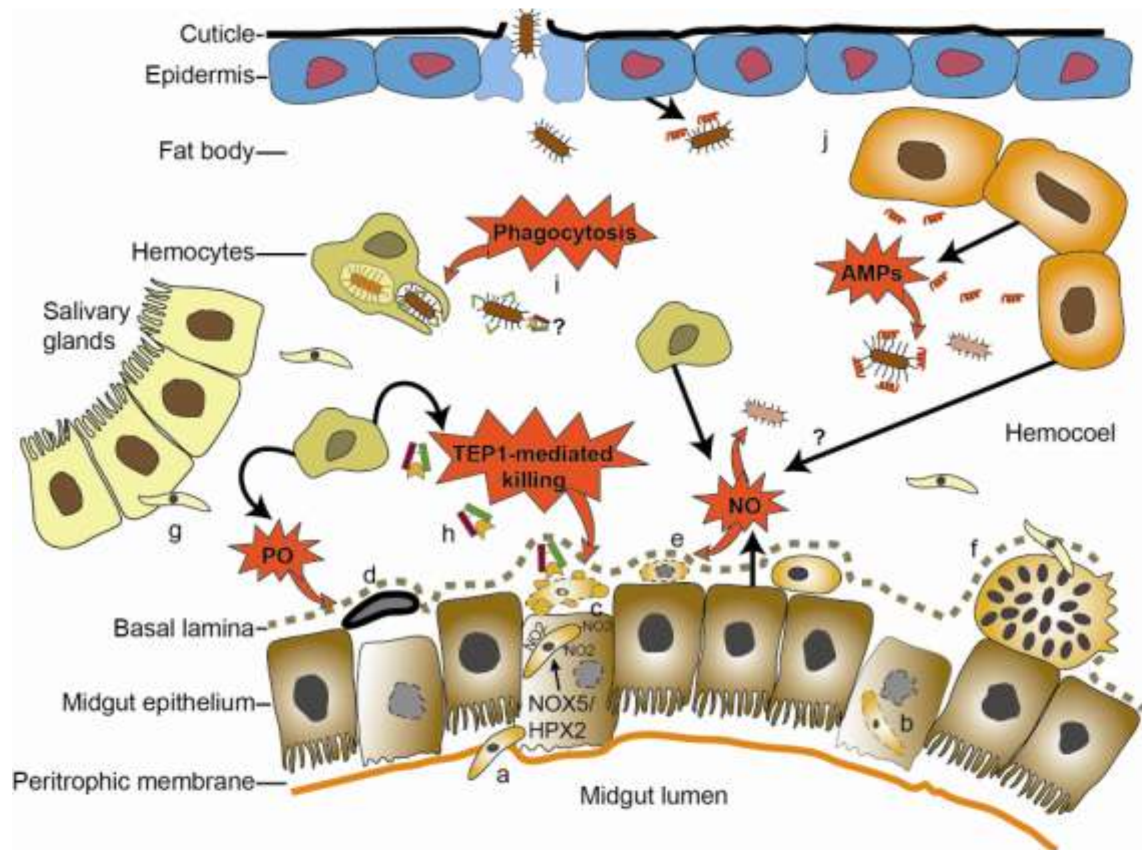


Figure 9. Immune effector mechanims in *Anopheles gambiae* innate immunity against invading *Plasmodium* parasites. (a) Ookinetes invade the midgut epithelium of *A. gambiae* 24hrs post-infection (b) Only few ookinetes are killed in the cytoplasm of the midgut epithelium through unknown mechanisms. (c) During invasion of epithelial cells, surface molecules on ookinetes become modified by reactive nitrogenous species through the joined activity of NOX5/HPX2 enzymes which render them susceptible to TEP1 mediated lysis when they emerge later to the basal labyrinth (d) Melanization in the mosquito basal labyrinth. (e) Early oocysts are killed by nitric oxide (NO) production by midgut epithelium cells and possibly by fat body. (f) Mature oocysts become resistant to killing, rupture 14 days post infection, releasing sporozoites into hemocoel. (g) These sporozoites migrate to and penetrate the salivary gland epithelium. (h) The LRIM1/APL1C/TEP1 present in the hemolymph mediates ookinete lysis (d) melanization of parasites and bacteria, and (i) phagocytosis of bacteria. (j) Systemic and local AMPs are produced by Fat body cells and barrier epithelial cells to help in defense against invading bacteria and possibly parasites (Modified from Yassine and Osta, 2010).

TEP1 is secreted by hemocytes into the hemolymph as a full length form (TEP-F, 165kDa) that seems to be spontaneously cleaved into a mature form ($TEP1_{cut}$, 80 kDa) (Levashina, *et al.,* 2001). $TEP1_{cut}$ is then stabilized by the LRIM1/APL1C complex and dislodged on the surface of the targeted microbe following recognition (figure 10). Silencing either LRIM1 or APL1C triggers the loss of $TEP1_{cut}$ from the hemolymph in naïve mosquitoes (Povelones, *et al.,* 2009; Fraiture, *et al.,* 2009). Different studies have shown that TEP1 (Blandin, *et al.,* 2004), LRIM1 (Osta, *et al.,* 2004) and APL1C (Riehle, *et al.,* 2006) all exhibit very similar RNAi phenotypes, characterized by increased numbers of oocysts in the gut. Also, TEP1, LRIM1, and APL1C are all required for the melanization response triggered against *Plasmodium* ookinetes in certain melanizing genotypes (Osta, *et al.,* 2004; Blandin, *et al.,* 2004; Povelones, *et al.,* 2009). Structurally, the mosquito LRIMs, including LRIM1 and APL1C, are characterized by having a coiled-coil domain, a cysteine pattern, and a signal peptide (Povelones, *et al.,* 2011). The APL1C/LRIM1 complex is thought to be a complement-control factor that carries TEP1 to the pathogenic surface. Yet, there is no clear indication that the complex interacts with microbial surfaces (figure 10). In addition to TEP1 stabilization by the APL1C/LRIM1 complex, more recent data revealed that TEP1 consumption during immune responses is also regulated by SPHs, namely SPCLIP1 and CLIPA2. SPCLIP1 was shown to co-localize with TEP1 on ookinete and bacterial surfaces whereby it enhances the conversion of TEP1-F to $TEP1_{cut}$, suggesting the existence of a complement convertase-like activity in the mosquito analogous to that operating in the mammalian immune system (Povelones, *et al.,* 2013). Interestingly, silencing LRIM1 in naïve mosquitoes triggers the loss of SPCLIP1 from the hemolymph, a phenotype similar to that observed in TEP1 kd, strongly suggesting that SPCLIP1 is a component of the TEP1 pathway. CLIPA2 was also shown to negatively

regulate the conversion of TEP1-F to $TEP1_{cut.}$ *CLIPA2 kd* enhances mosquito resistance to bacterial, fungal, and *Plasmodium* infections, possibly by enhancing $TEP1_{cut}$ accumulation on microbial surfaces. Interestingly, *LRIM1 kd* triggered the loss of CLIPA2 from the hemolymph of naïve mosquitoes suggesting that CLIPA2 is another component of the TEP1 pathway (Yassine, *et al.,* 2014). Hence, TEP1 seems to be under tight control not only at the transcriptional level by the NF-κB and JNK pathways, but also at the protein level by LRIMs and SPHs. This tight regulation highlights the key role of TEP1 in immunity and reveals that a defect in one of these regulatory mechanisms could entail an imbalanced immune response that may impact host fitness. Evidence for that was indeed shown in *CLIPA2 kd* mosquitoes which lay less eggs than their wildtype counterparts (Yassine, *et al.,* 2014).The role of CLIPA2 in complement regulation will be detailed in the results and discussion sections of my thesis. It is worth noting here that a recent evolution-based screen for genetic differentiation between *Anopheles* sister taxa identified selective sweeps of some immune genes, namely APL1C and TEP1, in two sister *Anopheles* species (*gambiae* and *coluzzi*) questioning whether this nucleotide variation, in turn, affects complement regulation among species (Mitri, *et al.,* 2015).

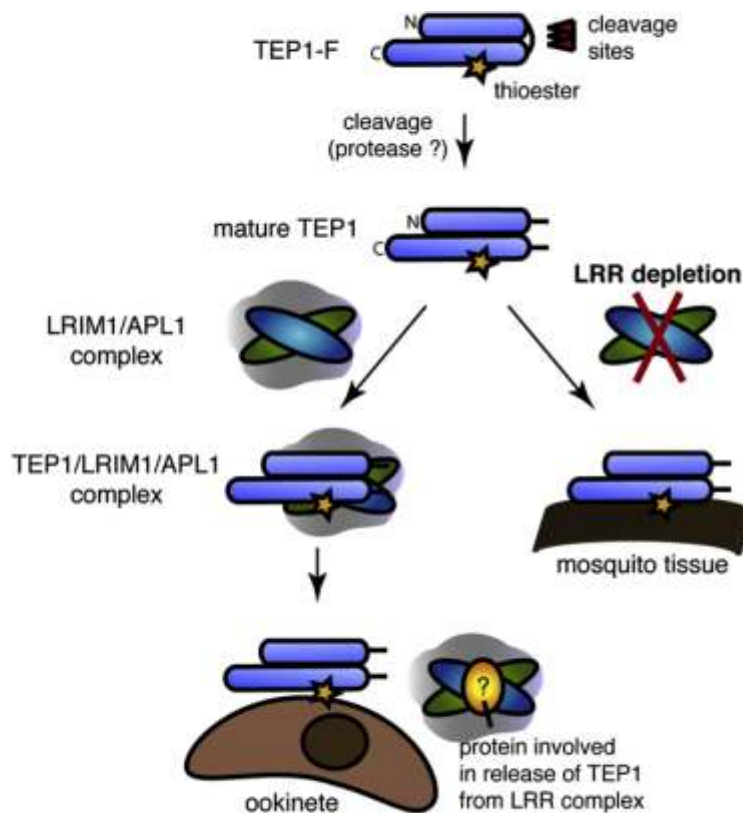


Figure 10. A Proposed model for TEP1 regulation through the LRIM1/ APL1C complex. In mosquito hemolymph, the inactive form of TEP1 (TEP1-F) is converted into a mature, active form ($TEP1_{cut}$). In $TEP1_{cut}$, the TEP1-N and TEP1-C fragments remain attached non-covalently. The LRIM1/APL1C complex, in turn, non-covalently binds to $TEP1_{cut}$ and controls its stability.Upon infection, $TEP1_{cut}$ is dislodged from the LRIM1/APL1C complex and deposited on parasitic surfaces through an unknown mechanism. When any of the two LRIMs is absent, $TEP1_{cut}$ is lost from circulation, deposits on self-tissues, and can no longer attack pathogenic surfaces.(Adapted Fraiture, *et al.,* 2009).

d. <u>Melanization</u>

Melanization is a potent immune response unique to arthropods. Besides its role in immunity, melanization is also required for cuticle sclerotization and wound healing. In large insects, melanization is thought to participate, in cooperation with hemocytes, in sequestering parasites in multicellular structures as well (Reviewed in Christensen, *et al.,* 2005). Yet, unlike encapsulation, for example, or other immune responses that require a direct participation of hemocytes, melanization is a humoral process that does not directly involve hemocytes (Reviewed in Christensen, *et al.,* 2005). Upon microbial

infection, PRRs recognize the invading pathogen by binding to its PAMPs, and activate downstream cascades of serine proteases involved in the proteolytic cleavage and conversion of the PPO zymogen into active PO. PO is the rate limiting step of melanization (Nappi and Christensen, 2005); it catalyzes the oxidation reaction of tyrosine into dopaquinone, which is then followed by a series of biochemical reactions, resulting in the synthesis and deposition of black-brown melanin pigments on the surface of invading pathogens (Soderhall and Cerenius, 1998; Schnitger,*et al*., 2007) (figures 11 and 12). The melanization immune response, however, has to be tightly regulated spatially to ensure the occurrence of melanogenesis directly on microbial surfaces and therefore prevent collateral damage of the host tissues. Despite the fact that melanization has been well studied in different insects at the biochemical and genetics levels, it remains unclear how PPO is localized to microbial surfaces and whether this entails its interaction with PRRs (figure 11). In *Drosophila* for instance, GNBP3 was shown to complex with PO in the hemolymph and could be involved in directing PO to fungal surfaces (Matskevich, *et al.,* 2010).

In *Drosophila* and *Anopheles gambiae*, the absence of *SPN43Ac*, also known as *necrotic*, (Levashina, *et al.,* 1999a; 1999b) and SPRN2 (Michel, *et al.,* 2005), respectively, resulted in the formation of spontaneous melanotic pseudotumors in adult tissues and in a noticeable reduction in the life spans of these flies and mosquitoes, suggesting that aberrant control of melanization imposes a fitness cost on the host. Melanization can target several microbes including parasites (Colins, *et al.,*1986), filarial worms (Christensen and Forton, 1986), bacteria (Nayar, *et al.,*1989), and fungi (Gotz, *et al.,* 1987; Bidocka and Khachatourians, 1987; Yassine, *et al.,* 2012); however, the contribution of melanization to immune defense against these model organisms remains controversial, and is likely to vary from one insect specie to another depending

on the composition of their microbial niche in the field that might dictate different investments in different arms of the immune response.

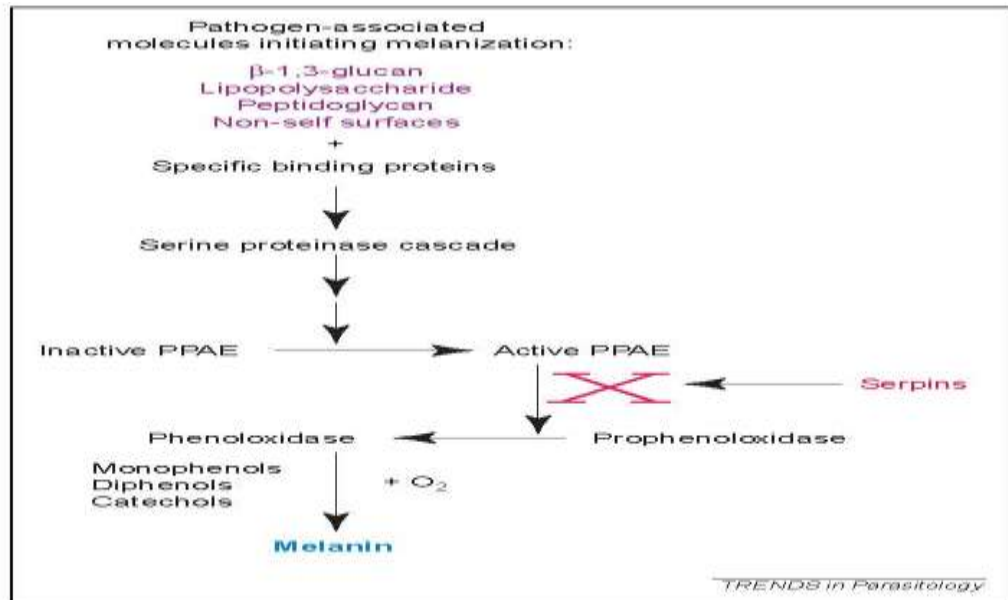


Figure 11. Schematic representation of the melanization activation cascade in insects. Recognition of pathogens promotes the activation of several downstream serine proteases resulting in PO activation and melanin deposition on the surface of the invading microbe (Adapted from Christensen, *et al.,* 2005).

i. The biochemical pathway of melanin biosynthesis in insects

In mammals, two distinct types of melanin are found: the brownish black eumelanin and the reddish yellow pheomelanin (Riley, 1997). In insects, however, only the brownish black eumelanin is detected. Enzymatically, melanin biosynthesis is initiated with the hydroxylation reaction of phenylalanine into tyrosine, in the presence of the phenylalanine hydroxylase (PAH) enzyme. In turn, tyrosine is hydroxylated into 3,4-dihydroxyphenyalanine (dopa) by the action of the enzyme PO, which is then oxidized into dopaquinone (Reviewed in Christensen, *et al.,* 2005). Dopaquinone subsequently undergoes nonenzymatic rearrangement and decarboxylation, generating dopachrome that generally reacts with thiol complexes (cysteine for example) to produce pheomelanin precursors. In insects however, a slight modification happens in

this pathway where the dopachrome conversion enzyme (DCE) acts on dopachrome, catalyzing its decarboxylation and resulting in the production of 5,6-dihydroxyindole (DHI) as the only product. DHI is then oxidized into indole-5,6-quinone, by the action of PO, and spontaneously polymerizes into brownish black eumelanin which is highly reactive and crosslinks with proteins located on microbial surfaces or at sites of injury (figure 12) (Reviewed in Christensen, *et al.,* 2005).

The *A .gambiae* genome reveals the presence of nine distinct PPO genes; six of which have distinctive temporal expression profiles during various developmental stages of the mosquito, suggesting a stage-specific contribution of the PO system to mosquito immunity/development. In the embryonic and larval stages for example, PPO1, PPO2, and PPO3 were shown to be highly transcribed. PPO4 transcripts, in turn, are mainly prominent in the transitional stage between late larval development-young pupae onset. In the late-pupae to adult stages, PPO5 and PPO6 exhibit high transcription profiles (Muller, *et al.,* 1999).

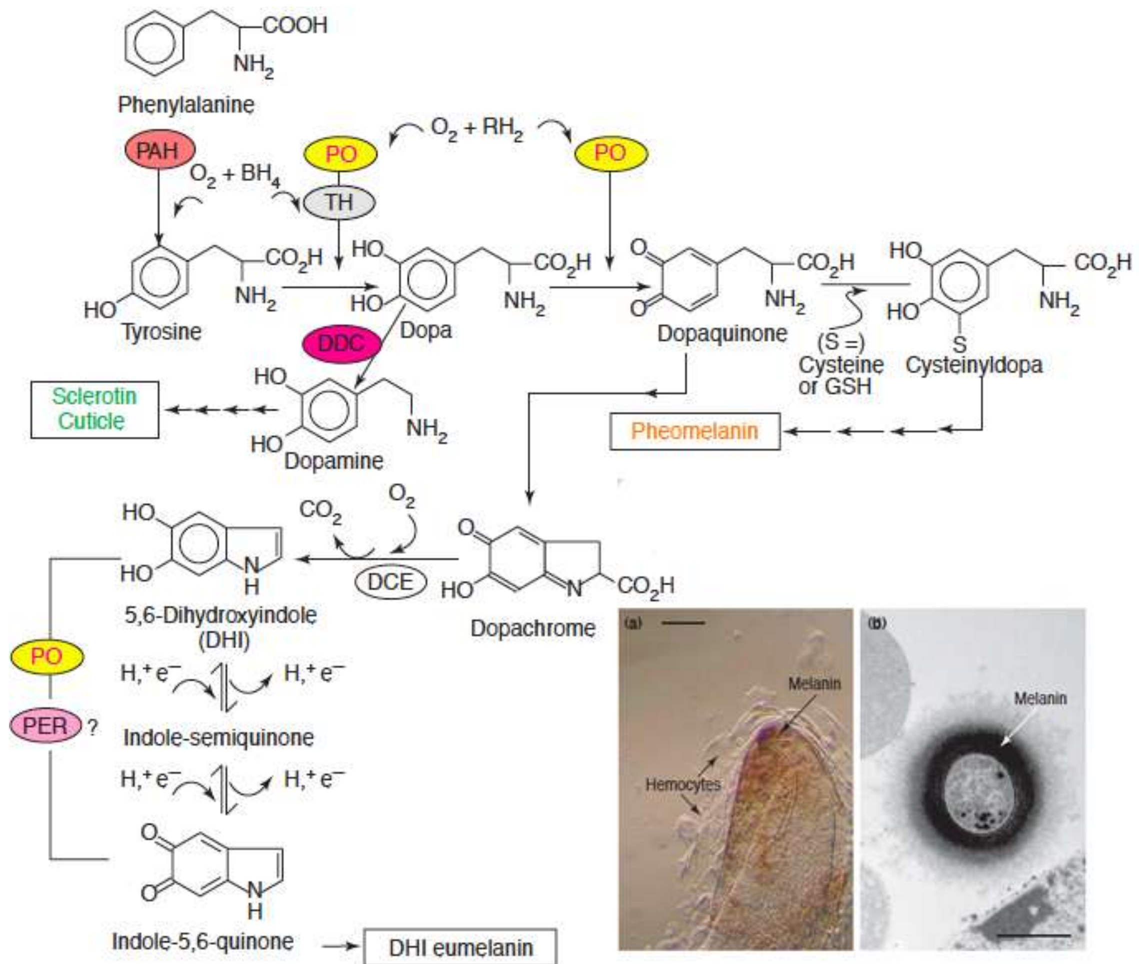


Figure 12. The biochemical cascade of melanogenesis. A scheme of the enzymatic reactions involved in melanogenesis. Two distinct examples of cell-mediated and humoral melanization are shown in the figure. (a) The parasitoid egg wasp *Leptopilina boulardi* is melanized and undergoes cellular encapsulation in the hemolymph of an infected fruit fly larvae (b) The bacteria *Enterobacter cloacae* is melanized in the hemolymph of adult *Aedes aegypti* mosquitoes in a humoral-mediated manner without any direct involvement of hemocytes (Adapted from Christensen, *et al.,* 2005).

e. Role of melanization in insect immunity

i. Anti-bacterial defense

The importance of melanization in anti-bacterial immunity seems to be controversial among different insect species. In the crayfish *Pacifastacus leniusculus*, for example, silencing PO enhances its susceptibility to the bacterial pathogen *Aeromonas hydrophila*, while knocking down pacifastin, a PO inhibitor, renders the

crayfish highly resistant to the pathogenic bacteria. This indicates an important contribution of melanization to the anti-bacterial defensive machinery of the crayfish and to its role in bacterial clearance (Liu, *et al.,* 2007). On the other hand, early genetic studies conducted in *Drosophila* and *Anopheles*, showed that melanization doesn't seem to be required for the resistance and survival of infected hosts to bacterial infections. In the fruit fly for instance, silencing either the MP1 or the MP2 proteases disrupted the melanotic cascade, yet, did not compromise the survival of infected flies to bacterial infection (Leclerc, *et al.,* 2006; Tang, *et al.,* 2006). Likewise, a study done in *A. gambiae* revealed that melanization seems to be dispensable for the mosquito anti-bacterial defense, despite the fact that bacteria injected directly into the hemocoel activate hemolymph PO (Schnitger, *et al.,* 2007). In a more recent study utilizing a broad panel of bacteria, *Drosophila* flies carrying a loss-of-function mutation in their clip domain serine protease MP2 that is required for hemolymph PO activity, showed compromised survival and resistance to both Gram-negative and Gram-positive bacterial infections (Ayres and Schneider, 2008). More recently, Lemaitre's group generated for the first time flies carrying deletion in PPO1 and PPO2, which are produced by larval crystal cells, but are also present in the adult flies hemolymph. In this study, it was shown that flies lacking both PPO1 and PPO2 succumb to Gram-positive and fungal infections, confirming that melanization plays non-redundant roles in the fly immunity (Binggeli, *et al.,* 2014). Collectively, these studies suggest that melanization contributes significantly to anti-bacterial defense in insects and that the discrepancies observed between different insect models and sometimes between different studies using the same insect model are due to the different strategies used to block the melanization response (mutations in upstream players such as CLIPs may not

completely block PO activity because of the ramification of pathways leading to PO) and the bacterial species/strains utilized to challenge insects.

ii. Anti-parasitic defense

So far, all *in vivo* functional genetic studies on *Plasmodium* melanization have been mainly done in the *A. gambiae-P .berghei* host-pathogen model system. In this system, *Plasmodium* melanization has only been observed in certain mosquito genotypes and has been either shown to be directly involved in ookinete killing (Volz, *et al.,* 2006), or in the clearance of dead parasites (Blandin, *et al.,* 2004; Shiao, *et al.,* 2006). However, melanization is rarely triggered by parasites infecting wild type laboratory strains of mosquitoes. Also, field caught mosquitoes rarely melanize malaria parasites (Niare, *et al.,* 2002). Moreover, the naturally melanizing refractory L3-5 *Anopheles* strain only melanizes ookinetes of certain *Plasmodium* species, including some strains of *P. falciparum*, but not sympatric strains of the human parasite (Collins, *et al.,* 1986). Also in melanizing mosquito genotypes such as *CTL4* kd and *SRPN2* kd, melanization was observed only against *P .berghei*, but not *P. falciparum* (Cohuet, *et al.,* 2006: Michel, *et al.,* 2006). A recent study revealed that a surface protein on *P. falciparum* ookinetes called Pfs 47 is an essential survival factor used by the parasite to evade the complement-like response in *A.gambiae*. Pfs 47 knock out parasites of the sympatric NF54 strain become completely melanized in L35 refractory mosquitoes, in a TEP1-dependent manner, unlike the wild type NF54 (Molina-Cruz, *et al.,* 2013), suggesting that the human parasites actively evades the mosquito immune response.

iii. Anti-fungal defense

Both vertebrates and invertebrates are able to sense β-(1,3) glucans of fungal cell walls using specific PRRs. Among arthropods, anti-fungal immunity has been mostly well studied in *Drosophila*. During the recognition process, the fungal β-(1,3) glucans are either sensed by the soluble β-glucan recognition receptor GNBP3, or by the CLIP persephone which detects the proteolytic activity of different fungal virulence factors, namely the *B. bassiana* protease PR1 (Gottar, *et al.,* 2006). This recognition, in turn, triggers the intitation of a serine protease cascade that promotes the cleavage of spaetzle and thereby activates the Toll pathway, leading to the synthesis of the drosomycin and metchnikowin anti-fungal AMPs (Hetru, *et al.,* 2003). GNBP3 was also shown to trigger anti-fungal immunity in a Toll-independent manner. In this scenario, GNBP3 promotes the agglutination of fungal cells and assembles immune effector complexes containing PO leading to fungal melanization, suggesting that melanization plays an important role in anti-fungal immunity (Matskevich, *et al.,* 2010). A recent study from Lemaitre's lab revealed that adult flies lacking PPO1 and PPO2 succumb to diverse fungal infections, providing definite evidence for the importance of this immune process in anti-fungal defense. In the context of this thesis work, we have shown that by silencing CLIPA8, a key positive regulator of the melanization response, mosquitoes become more susceptible to fungal infections (Yassine, *et al.,* 2012). In *Aedes aegypti*, CLSP2, a modular serine protease containing a CTL lectin domain, recognizes the invading fungus and acts as a key modulator of mosquito anti-fungal immunity. Upon *B. bassiana* infection, CLSP2 is cleaved, and exposes its CLSP2 CTL domain that binds to the β-1,3-glucans in fungal cell wall. *CLSP2* kd mosquitoes are more susceptible to fungal infection, providing additional evidence for the importance of melanization in anti-fungal immunity in mosquitoes. In this study as well, CLSP2 was shown to

modulate a large panel of innate immunity genes involved in the Toll pathway and in the melanization cascade in mosquitoes (Wang, *et al.,* 2015).

5. *Cellular anti-microbial immunity other than phagocytosis*

a. Cellular encapsulation

Although the process of cellular encapsulation is still not clearly understood in insects, some studies showed the involvement of insect lamellocytes in the formation of multilayered capsules that surround the invading microbe leading to its killing through melanization, asphyxiation, and secretion of free radicals (Gotz, *et al.,* 1986).

b. Epithelial nitration and production of reactive oxygen species

Malaria parasites invading the midgut promote the expression of nitric oxide synthase (NOS), heme peroxidase 2 (HPX2), and hydrogen peroxide generating enzyme NADPH oxidase 5 (NOX5), three enzymes that cooperate to catalyze protein nitration in infected midgut epithelial cells (Han, *et al.,* 2000; Kumar, *et al.,* 2004; Oliveira, *et al.,* 2012). Silencing either *HPX2* or *NOX5* results in a dramatic increase in the number of *P. berghei* oocysts in the midgut of infected mosquitoes, implying a significant anti-*Plasmodium* role for the HPX2/NOX5 system, which exhibits its effects through the production of reactive nitrogen species such as nitric oxide and peroxynitrites (Oliveira, *et al.,* 2012). Interestingly, nitration of ookinete surface proteins seems to be a pre-requisite for TEP1-mediated complement activity when parasites egress from cells into the basal labyrinth of the midgut epithelium. Nitration is likely to introduce chemical modifications to the parasite surface proteins rendering them more "visible" to the immune system. This coordinated response between epithelial nitration and the complement-system seems to be controlled by the JNK pathway. This pathway limits

Plasmodium infection by activating caspase-9 and promoting the expression of HPX2 and NOX5 enzymes which potentiate epithelial nitration, and up regulate the two effector immune genes *TEP1* and *FBN9* (Garver, *et al.,* 2013; Ramphul *et al.*, 2015). It is worth noting here that this proposed model seems to be highly dependent on the rate of midgut invasion; epithelial nitration and complement-mediated killing may be evaded if ookinetes rapidly and transiently invade cells (Oliveira, *et al.,* 2012).

c. <u>Actin polymerization</u>

In *A. gambiae*, the contribution of actin-based cytoplasmic protrusions to cellular anti-*Plasmodium* defense has been primary studied using *in vivo* real time analysis of ookinete locomotion during mosquito midgut invasion. In these studies, it was shown that cytoplasmic protrusions composed of actin extending from the invaded midgut epithelial cells surround *P. berghei* ookinetes and form “hoods” around those parasites exiting from the basal side of midgut epithelial cells (Vlachou, *et al.,* 2004). In fact, among the genes that are highly activated in response to ookinete invasion of the midgut are those encoding for proteins mainly implicated in regulating the actin-cytoskeleton dynamics (Vlachou, *et al.,* 2005). Silencing *WASP*, an actin-cytoskeleton remodeling factor, resulted in a dramatic increase in the number of *P. berghei* (Vlachou, *et al.,* 2005) and *P. falciparum* (Mendes, *et al.,* 2008) oocysts in the midguts of infected mosquitoes. In a recent study, secreted *A. gambiae* actin5c was also shown to act as a *Plasmodium* antagonist, and globular and filamentous actin were shown to exhibit distinct extracellular immune functions against both parasites and bacteria (Simoes and Dimopoulos, 2015).

On the other hand, a study proposed that actin polymerization is basically associated with melanization whereby both reactions are involved in the wound healing

processes that target and isolate dying/dead parasites perceived as damaged tissues (Shiao, *et al.,* 2006). It was shown that these dying/dead ookinetes in the mosquito extracellular spaces are surrounded by cytoplasmic extensions known as "organelle-free actin zone" (AZ) that strongly correlate with ookinete melanization in the L35 refractory mosquito strain. Silencing the actin cytoskeletal remodeling factors frizzled-2 (Fz2) and cell division cycle 42 (Cdc42) did not seem to affect ookinete development, although AZ construction and melanization were completely aborted in these mosquito genotypes (Shiao, *et al.,* 2006). This discrepancy in the contribution of actin polymerization to anti-parasite immunity necessitates further studies.

6. The role of lipid carriers in mosquito immunity

Lipophorin (Lp) is a major lipid carrier molecule in insects. Its mode of lipid delivery through the hemolymph to various organs resembles a reusable shuttle, retaining the protein matrix of the Lp particle with no further degradation post delivery (Raikhel, *et al.,* 2006). Structurally, Lp consists of three apolipoproteins: apolipoprotein I and apolipoprotein II encoded by a single gene (Kutty, *et al.,* 1996 and Babin, *et al.,* 1999), as well as apolipoprotein III which is the exchangeable part of the complex (Law and Wells, 1989, Arrese, *et al.,* 2001). The intracellular uptake of Lp by fat body and ovary cells happens through its associated lipophorin receptor (LpR), which belongs to the low density lipoprotein receptors (LDLRs) superfamily (Babin, *et al.,* 1999; Van Hoof, *et al.,* 2002, 2003; Choe, *et al.,* 2001, Seo, *et al.,* 2003). In *A. aegypti*, two splice variants of the *LpR* gene product specific to the fat body (*AaLpRfb*) and ovary (*AaLpRov*) have been identified indicating a possible distinctive role of Lp in mosquito reproduction in addition to its well established role in lipid metabolism (Choe, *et al.,* 2001, Seo, *et al.,* 2003). Generally, *AaLpRfb* transcript expression happens in the

postvitellogenic period, where fat bodies are transformed into lipid, carbohydrate and protein storage depots (Seo, *et al.,* 2003); yet the expression of most genes in the fat body is regulated during the vitellogenesis period in the presence of a blood meal-driven hormonal cascade (Raikhel, *et al.,* 2002). Functionally, in addition to the role of Lp in lipid metabolism and reproduction, Lp was shown to play important roles in immunity to infectious diseases (Bansal, *et al.,* 2005). In both invertebrates and mammals for example, lipid carrier molecules contribute to lipopolysaccharide detoxification (Kato, *et al.,* 1994; Feingold, *et al.,* 1995; Kitchens, *et al.,* 1999). Lipophorins were also shown to be components of the wound-clotting response in *Periplaneta* (Coodin and Caveney, 1992) and in *Galleria* (Mandato, *et al.,* 1996). Additionally, ApoIII is involved in various immunological responses including the activation of hemolymph lytic activity, the activation of prophenoloxidase, the expression of antimicrobial peptides, among others (Halwani and Dunphy, 1999; Halwani, *et al*., 2000; Dettloff, *et al.,* 2001; Mullen and Goldsworthy, 2003; Kim, *et al*., 2004). A recent study showed that pathogenic infections (Gram-negative and fungus) and *Plasmodium gallinaceum* parasitic infections promote the up-regulation of *Lp* and *LpRfb* gene expression in infected *A. aegypti* mosquitoes. In *Lp* kd *A. aegypti* mosquitoes, the number of developing *Plasmodium* oocysts was reduced by 90% (Shin, *et al*., 2006). In *A. gambiae*, two nutrient transporting proteins Lp and vitellogenin (Vg) were shown to reduce TEP1 anti-parasitic efficiency. In the absence of any of these two proteins, TEP1 binding to ookinete surfaces becomes more efficient leading to more ookinete killing. In this study as well, it was shown that the normal expression of Vg and the growth of *Plasmodium* oocysts both require Lp (Rono, *et al*., 2010). The contribution of lipophorin to *A. gambiae* immunity and its regulation of TEP-1 mediated immune defense were

extensively investigated in the context of my thesis and will be detailed in the result section.

7. The contribution of the midgut microbial flora to Anopheles immunity

The midgut microbial flora seems to have mostly an indirect role in modulating the numbers of *Plasmodium* parasites that successfully develop in infected mosquitoes. In two independent studies, aseptic antibiotic treated *A. gambiae* mosquitoes lacking their midgut microbial flora had larger numbers of *P. berghei* and *P. falciparum* oocysts in their midguts, as compared to septic ones (Dong, *et al.*, 2009; Meister, *et al.*, 2009). Noticeably, the bacterial microbial flora seems to be implicated in anti-parasitic immunity by exerting its effect mainly at the ookinete stages (Dong, *et al.*, 2009). These commensal bacteria proliferate dramatically in the midgut lumen after a blood meal, stimulating the basal levels of many immunity genes, including *lysozyme*, *AMPs*, *CLIPs*, *lectins* and *TEPs*, hence priming mosquito immunity, and thereby rendering the mosquito more resistant to *Plasmodium* infections (Meister, *et al.*, 2009; Dong, *et al.*, 2009). Besides this indirect role of the bacterial microbial flora in controlling mosquito resistance to *Plasmodium* infections, it was shown that some bacterial species, like *Enterobacter* for example, promote the generation of reactive oxygen species that directly interfere with *Plasmodium* stages present in the gut lumen before it invades the mosquito midgut (Cirimotich, *et al.*, 2010).

The impact of the gut microbiota on *Plasmodium* development fueled the interest in using specific species of this flora, especially those that are vertically transmitted, in paratransgenic approaches that aim to kill the parasites in the mosquito blocking their transmission to humans. Some bacterial species that live in symbiosis with mosquitoes like the acetic acid bacteria of the genus *Asaia* were also shown to

colonize the female eggs and the male reproductive system of several human mosquito vectors including *Anopheles gambiae*, *Anopheles stephensi*, *Aedes Albopictus*, and *Aedes aegypti*. *Asaia* are vertically transmitted from mother to offsprings, thereby rapidly colonizing mosquito populations (Chouaia, *et al*., 2010; Crotti, *et al*., 2009;Damiani, *et al*., 2010; Favia, *et al*.,2007; 2008). *Pantoea agglomerans*, another example of a common mosquito symbiont bacterium, was engineered to synthesize several anti-*Plasmodium* effector proteins and secrete them via the *E.coli* hemolysin A secretion system. This *P. agglomerans* strain interfered in the development of malaria parasites and resulted in 98% reduction in the numbers of both *P. berghei* and *P.falciparum* (Wang, *et al*., 2012).

The endosymbiont wMelPop strain of *Wolbachia* that is also maternally inherited is another interesting candidate whose introduction into *Aedes aegypti* up-regulated mosquito immunity resulting in the inhibition of the dengue virus replication (Walker, *et al*., 2011) and the inhibition of the development of filarial nematodes, in addition to shortening the life span of *Aedes* (Kambris, *et al*., 2009). Transient introduction of wMelPop into *A. gambiae* significantly reduced *Plasmodium* infection intensity by up-regulating some immune genes such as TEP1 (Kambris, *et al*., 2010). Although attempts to introduce wMelPop in *A. gambiae* failed, recent analysis of field mosquitoes revealed natural *Wolbachia* infections of *A. gambiae*, and that these strains are vertically transmitted, opening up to the potential use of these bacteria to limit malaria transmission (Baldini, *et al*., 2014). Also, paratransgenic approaches involved the use of entomopathogenic fungus like *Metarhizium anisopliae* expressing either a single chain antibody that agglutinates sporozoites, an SM1 peptide that blocks the attachment of parasite sporozoites to the mosquito salivary glands, or the antimicrobial peptide scorpine, resulted in significant reduction in the number of *P. falciparum*

sporozoites by 85%, 71%, and 90%, respectively, in these fungal infected *A. gambiae* mosquitoes (Fang, *et al*., 2011).

CHAPTER II

AIMS OF THE PROJECT

Genetic studies in *Anopheles gambiae* highlighted the important role of the mosquito immune system in limiting the numbers of malaria parasites that successfully develop within the vector and led to the identification of key immunity genes belonging to distinct genes families. Among these are clip domain serine proteases (CLIPs) that play central roles in insect immunity. Functionally, CLIPs are divided into two groups; the catalytic CLIPs that drive the enzymatic cascades in the mosquito hemolymph and the non-catalytic CLIPs which are proposed to act as regulators of these cascades. Non-catalytic CLIPs, also known as serine protease homologs (SPHs), have been mainly implicated in the regulation of the melanization immune response in several insect species (Kim, *et al.*, 2002; Schnitger, *et al.*, 2007; Yu, *et al.*, 2003); however their mechanisms of action and their broader role in immunity remain poorly investigated. The main research interest of my thesis project is to understand the role of CLIPA2, one of these SPHs, in mosquito immune responses to systemic infection, particularly that caused by entomopathogenic fungi such as *B. bassiana*.

In *A. gambiae*, CLIPA2 was identified through a systematic functional genetic screen and found to act as a negative regulator of the mosquito melanization response against *Plasmodium* ookinetes (Volz, *et al.*, 2006). However, it remained unclear whether CLIPA2 exhibits a broader role in mosquito immunity that includes also bacterial and fungal systemic infections. I was particularly interested in studying its role against systemic fungal infections using *B. bassiana* as a model entomopathogenic fungus since fungi constitute one of the most important threats to mosquitoes in nature, as they invade the mosquito hemocoel through the cuticle, bypassing the oral route utilized by malaria parasites and bacteria to infect the host. In past studies, most fungal

infections of mosquitoes were usually done by injecting spores directly into the abdomen which is an artificial mode of infection or by rolling mosquitoes on a plate containing fungal spores, a protocol adapted from *Drosophila*. However, the latter often caused infection-independent mortalities in mosquitoes which tend to lose their legs due to electrostatic interactions during the rolling process. Hence, there was a need to establish a robust protocol for infecting mosquitoes with fungi that mimics natural infections in the field and that avoids collateral damage to the host resulting from the infection process itself. In **specific aim 1** of my project, I worked on designing a method for infecting adult mosquitoes with *B. bassiana* that is gentle on the host and produces reliable results. I tested the efficiency of this method by comparing the susceptibility of mosquitoes infected with WT *B. bassiana* with those infected with transgenic fungi expressing the trypsin modulating oostatic factor (TMOF) hormone. Having a consistent and reliable method of fungal spores delivery in hand; in **specific aim 2** of my project I studied the role of melanization in defense against the entomopathogenic fungus *Beauveria bassiana* by temporally monitoring the development of GFP-expressing fungi in *Anopheles*, and by scoring for melanin deposition on these fungal hyphae during different fungal developmental stages. In this study as well, I investigated whether *CLIPA8* and *TEP1*, two important positive regulators of melanization, are potentially recruited to fungal hyphae and whether these two genes contribute to *Anopheles* tolerance and resistance to fungal infection. In **specific aim 3** of my project, I mainly focused on characterizing the role of CLIPA2 in mosquito immune responses to systemic fungal and bacterial infections by performing an in-depth functional and molecular analysis of CLIPA2. I investigated whether CLIPA2 is potentially recruited to microbial surfaces and whether it contributes to mosquito tolerance and resistance to pathogenic infection. I particularly focused on

identifying proteins interacting with CLIPA2 and subjecting selected candidates to *in vivo* functional genetic analysis in order to draw a comprehensive functional model of the CLIPA2 interactome and to shed more light on the regulatory role of CLIPA2 in mosquito immunity. The signaling pathways controlling the expression of CLIPA2 following microbial infections were also investigated in the context of the third aim of my thesis work.

Despite the significant progress in our understanding of the roles and mechanisms of action of many immune effector mechanisms in mosquito, building a detailed comprehensive module of these mechanisms is still far beyond reach. Collectively, this work is expected to promote our knowledge in mosquito immunity, in particular, and in insect immunity, in general, and would hopefully help in deciphering the interplay that exists between these different immune effector mechanisms.

CHAPTER III

PREFACE TO CHAPTER IV

The following chapter consists of four parts, with each part presenting the results of one study. In the first study (Expression of trypsin modulating oostatic factor (TMOF) in an entomopathogenic fungus increases its virulence towards *Anopheles gambiae* and reduces fecundity in the target mosquito), I have included a published manuscript in *Parasites and Vectors* (Kamareddine, *et al.,* 2013) after reformatting it to match the desired style of the dissertation. Yanhua Fan, a member of Dr. Nemat kehani's working group at University of Florida, produced the transgenic fungus expressing TMOF, and I have conducted all the experiments in this study. In the second study (The mosquito melanization response is implicated in defense against the entomopathogenic fungus *Beauveria Bassiana*), I have also included the published manuscript in *PLoS Pathogens* (Yassine, *et al.,* 2012) which was further formatted as required. Both Hassan Yassine and I equally contributed to this work. My contributions were figures 15, 16, 17, 18, 19, 21, 22, and 23. In the third study, (A serine protease homolog negatively regulates TEP1 consumption in systemic infections of the malaria vector *Anopheles gambiae*), I also formatted and included the published paper in *Journal of Innate Immunity* (Yassine, *et al.,* 2014). My contributions to this study were figures 24, 25, 26, and 29. The results of my forth study, (Functional interaction between apolipophorins and complement regulate the mosquito immune response to systemic infections), were also formatted to match the dissertation style and included in an accepted manuscript in *Journal of Innate Immunity* (Kamareddine, *et al.,* 2016), which is still in press. I have contributed the following figures: 31, 32A, 33, 34, 35, 36, S2, S3, and tables. The rest of the Figures were contributions of my colleague Johnny Nakhle.

CHAPTER IV

RESULTS

A. Expression of trypsin modulating oostatic factor (TMOF) in an entomopathogenic fungus increases its virulence towards *Anopheles gambiae* and reduces fecundity in the target mosquito

1. Abstract

Background: Adult and larval mosquitoes regulate food digestion in their gut with trypsin modulating oostatic factor (TMOF), a decapeptide hormone synthesized by the ovaries and the neuroendocrine system. TMOF is currently being developed as a mosquitocide, however, delivery of the peptide to the mosquito remains a significant challenge. Entomopathogenic fungi offer a mean for targeting mosquitoes with TMOF.

Findings: The efficacy of wild type and transgenic *Beauveria bassiana* strains expressing *Aedes aegypti* TMOF (*Bb*-Aa1) were evaluated against larvae and sugar and blood fed adult *Anopheles gambiae* mosquitoes using insect bioassays. *Bb*-Aa1 displayed increased virulence against larvae, sugar fed (sf) and blood fed (bf) adult *A. gambiae* when compared to the wild type parent strain. Median lethal dose (LD50) values decreased by ~20% for larvae, and ~40% for both sugar and blood fed mosquitoes using *Bb*-Aa1 relative to the wild type parent. Median lethal time (LT50) values were lower for blood fed compared to sugar fed mosquitoes in infections with both wild type and *Bb*-Aa1. However, infection using *Bb*-Aa1 resulted in 15% to 25% reduction in LT50 values for sugar- and blood fed mosquitoes, and ~27% for larvae, respectively, relative to the wild type parent. In addition, infection with *Bb*-Aa1 resulted in a dramatic reduction in fecundity of the target mosquitoes.

Conclusions: *B. bassiana* expressing *Ae. aegypti* TMOF exhibited increased virulence against *A. gambiae* compared to the wild type strain. These data expand the

range and utility of entomopathogenic fungi expressing mosquito-specific molecules to improve their biological control activities against mosquito vectors of disease.

2. Introduction

Mosquito vectors transmit numerous diseases to humans and animals, causing illness and death that result in huge socio-economical burdens, especially in endemic countries. Control of mosquito vector populations has been almost exclusively based on the use of insecticidal chemicals; however, the strong dependence on insecticides for mosquito control worldwide and the use of such chemicals in agriculture has led to the physiological resistance of important mosquito vectors in recent years (Koekemoer, *et al.*, 2011; Labbe, *et al.*, 2007; Lima, *et al.*, 2011). Entomopathogenic fungi, such as *Metarhizium anisopliae* and *Beauveria bassiana*, both EPA approved biological control agents (http://www.epa.gov/pesticides/), offer an environmentally friendly alternative to chemical insecticides, are virulent to mosquitoes, and have been considered as possible candidates for reducing disease transmission by insect vectors (Blandford, *et al.*, 2005; Read, *et al.*, 2009; Scholte, *et al.*, 2004). Despite their potential, widespread use of these fungi remains limited, due to a number of factors that include slow killing speed, low resistance to abiotic stress, issues of spore viability, and the amount of material (spores) needed for effective control. Nevertheless, there is still significant interest in using these biological control agents due to their effectiveness against insecticide resistant mosquito species (Howard, *et al.*, 2011) and advances in the development of tools and lures for their delivery to mosquitoes (Bukhari, *et al.*, 2011). Several attempts at increasing the insecticidal effectiveness of entomopathogenic fungi have been based on the development of recombinant DNA techniques. In this context, transgenic entomopathogenic fungi were developed that express potent insect-specific neurotoxins

that kill the infected host (Wang and Jiang, 2007), or enzymes that enhance fungal resistance to adverse conditions such as UV (Fang and St.Leger, 2012), ultimately increasing spore viability in nature. In addition to increasing their insecticidal potential, entomopathogenic fungal strains can also be manipulated to target the pathogen, e.g. *Plasmodium* itself within its mosquito vector (Fang, *et al.*, 2011). Recently, the idea of using targeted host-specific molecules to subvert normal insect development has been proposed (Fan, *et al.*, 2011; Keyhani, 2012). Insects synthesize hormones and neuropeptides that have been exploited as targets for pest control. Examples of these include diuretic hormones and trypsin modulating oostatic factors (TMOFs) that are found in insects including mosquitoes and flies. Administration of diuretic hormone increased secretion of fluids from the malphigian tubules leading to water loss and death of the insect. The *Ae. aegypti* TMOF (*Aea*-TMOF) was shown to circulate in the hemolymph, bind to gut receptors and inhibit trypsin biosynthesis by exerting a translational control on trypsin mRNA (Borovsky, *et al.*, 2006). TMOF is resistant to proteolysis in the gut and easily traverses the gut epithelial cells into the hemolymph in adults and larvae, hence, it can be fed to mosquitoes resulting in inhibition of food digestion, anorexia, and is ultimately lethal to the insect (Borovsky, 2003; Borovsky, *et al.*, 1993; Borovsky and Meola, 2004; Bylemans, *et al.*, 1994). In this report, we measured the virulence of a *B. bassiana* strain expressing *Aea*-TMOF to adult (sugar and blood fed) and larval stages of *Anopheles gambiae*, the major malaria vector in Africa. The effect of this strain on mosquito fecundity was also determined.

3. Results

B. bassiana strain *Bb*-Aa1 expresses *Aea*-TMOF as a fusion protein with a 28 amino acid signal peptide derived from the *B. bassiana* chitinase gene to drive the

extracellular secretion of the hormone (Fan, *et al*., 2012). Sugar and blood fed adult female *A. gambiae* as well as larvae were exposed to spores (conidia) of strain *Bb*-Aa1 or the wild type parent to determine the effect of *Aea*-TMOF expression on virulence. *Bb*-Aa1 was more potent than its wild type parent against both sugar and blood fed adults (Figure 13A and 13B), causing 40% reduction in LD50 values (50% mortality) in both groups compared to the wild type control (Table 1). However, LD50 values were similar between sugar and blood fed mosquitoes infected with the same fungal strain, regardless of its type (*Bb*-Aa1 or wild type). Infection with *Bb*-Aa1 also induced a 15 and 25% reduction in the mean survival times (LT50 values) of sugar and blood fed mosquitoes, respectively, compared to the wild type strain. LT50 values were also lower for blood- compared to sugar fed mosquitoes infected with the same strain, regardless its type. The fact that infections with the same strain, irrespective of its type, resulted in similar LD50 values between sugar and blood fed mosquitoes, but lowered consistently the LT50 values for the blood compared to sugar fed group, suggests that the blood meal itself does not seem to affect the virulence of a particular *B. bassiana* strain but rather mosquito tolerance to infection. Our data are in contrast to Mnyone *et al.* who reported that blood fed mosquitoes tended to be similar to or displayed greater median survival times than their unfed cohorts. There are several possible explanations for this discrepancy that can include the assay conditions (Mnyone et al. used 2×10^{10} conidia/m^2 formulated in an oil suspension and sprayed onto sheets) and strain variation (whether fungal or mosquito), and this deserves greater attention.

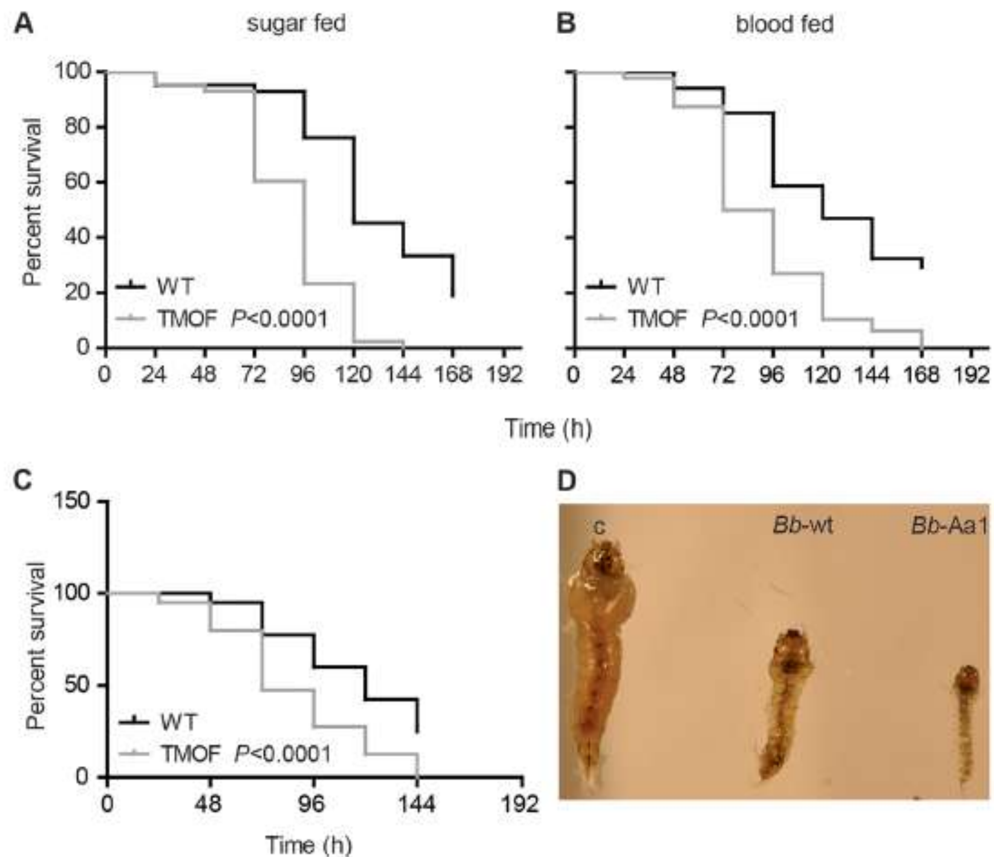


Figure 13. Expression of *Aea*-TMOF increases *Beauveria bassiana* virulence to *Anopheles gambiae* mosquitoes. (A) Sugar fed and (B) blood fed adult female *A. gambiae* mosquitoes were challenged with *Bb*-Aa1 strain expressing *Aea*-TMOF or the wild type parent, by spraying with a suspension of $1x10^8$ spores/ml and their survival was scored daily over the indicated period. (C) Three day old larvae were challenged by applying 5.5 x 10^8 spores (suspended in 50 µl oil) to the water surface and their survival was scored daily over the indicated period as described in the Methods section. Graphs represent percent survival as calculated by the Kaplan-Meier method for one representative experiment of each group. Statistical significance was calculated by the log rank test and survival curves were considered significantly different if $P<0.05$. (D) Six days after *B. bassiana* infection, larvae showed significant growth retardation compared to non-infected controls. Wild type *B. bassiana* (*Bb*-wt); TMOF expressing strain (*Bb*-Aa1). Data shown are representative of at least three independent biological replicates using different batches of mosquitoes and conidia.

Anopheles gambiae larvae infected with *Bb*-Aa1 exhibited greater compromised growth and survival as compared to those infected with the wild type strain (Figure 13C). *Bb*-Aa1 infection of larvae resulted in 20 and 27% reductions in

LD50 and LT50 values, respectively, compared to the wild type strain (Table 1). Six days after *B. bassiana* infection, larvae exhibited significant growth retardation evidenced by their abnormally small size compared to non-infected controls; however, the phenotype was more severe in *Bb*-Aa1 infected larvae compared to those infected with the wild type strain (Figure 13D).

Table 1 LD_{50} and LT_{50} values of wild type and *Aea*-TMOF expressing *Bb*-Aa1 strain against *Anopheles gambiae*

LD_{50} and LT_{50} values of wild-type and TMOF expressing *B. bassiana* infections in *Anopheles gambiae*

Strain	Mosquito stage	LD_{50} (conidia/ml)	LT_{50} (h)
Wild-type *B. bassiana* (sf)	Adult	$18.34 \pm 2.86 \times 10^{7a}$	133.26 ± 12.79^{b}
Bb: spAeaTMOF (sf)	Adult	$11.08 \pm 2.79 \times 10^{7a}$ (*P*=0.01)	113.23 ± 17.56^{b} (*P*=0.009)
Wild-type *B. bassiana* (bf)	Adult	$18.00 \pm 2.01 \times 10^{7c}$	115.04 ± 11.26^{d}
Bb: spAeaTMOF (bf)	Adult	$11.31 \pm 0.01 \times 10^{7c}$ (*P*= 0.04)	86.48 ± 4.68^{d} (*P*=0.01)
Wild-type *B. bassiana*	Larvae	$14.07 \pm 1.24 \times 10^{7e}$	116.31 ± 13.69^{f}
Bb: spAeaTMOF	Larvae	$11.32 \pm 0.77 \times 10^{7e}$ (*P*=0.01)	85.05 ± 14.66^{f} (*P*=0.001)

[a,c] LD_{50} calculated from the 76 h time point.
[e] LD_{50} calculated from the 48 h time point using the Probit analysis.
[b,d] Bioassays performed by spraying mosquitoes with a concentration of 1×10^{8} spores/ml.
[f] Larval bioassays performed using a concentration of 5×10^{6} spores/ml.
Statistical analysis was performed used the Student's T-test and values were considered significant if $P<0.05$.

Egg laying was significantly affected by fungal infection. Infection of *A. gambiae* mosquitoes with the wild-type *B. bassiana* strain resulted in a significant reduction (~16%) in fecundity compared to non-infected controls (Figure 14). However, expression of Aea-TMOF resulted in a dramatic reduction (~60%) in fecundity compared to controls (Figure 14). These data suggest that strain *Bb*-Aa1 also has the potential to reduce the size of *A. gambiae* mosquito populations by severely

compromising fecundity. *Aea*-TMOF does not induce vertebrate toxicity (Borovsky, 2007) and has passed EPA approval (http://www.epa.gov/ pesticides/). Laboratory bioassays showed that the hormone is effective against all the major species of mosquitoes including Anopheline (Borovsky and Meola, 2004). Data from this as well as a previous study (Fan, *et al*., 2012) indicate that expressing *Aea*-TMOF in entomopathogenic fungi is a promising approach for the delivery of the hormone to mosquitoes.

Entomopathogenic fungi strongly affect mosquito survival (Blandford, *et al*., 2005; Mnyone, *et al*., 2012; Scholte, *et al*., 2005) and may constitute a reliable measure for mosquito control when used as part of an integrated vector management plan. A field trial in a malaria endemic village in Tanzania, using experimental huts that simulate local houses, revealed that certain applications of fungal-treated surfaces lead to the infection of approximately 70% of mosquitoes entering the hut, which according to their model, is expected to reduce malaria transmission by 75-80% (Mnyone, *et al*., 2012). Of particular relevance to the data presented here, their model predicts that maintaining a high reduction in malaria transmission rates at lower levels of infected mosquitoes necessitates an increase in fungal virulence (Mnyone, *et al*., 2012). Hence, strains like *Bb*-Aa1 with improved virulence over standard parent strains are expected to strongly reduce malaria transmission even when the prevalence of fungal-infected mosquitoes is moderate. Fungal strains with a narrower host range and coupled to specific TMOF peptides can also be constructed to maximize specificity and minimize non target effects. TMOF is a physiologically important host hormone that must be expressed during the life stages of adults and larvae to control digestion and growth (Borovsky, 2003), thus, the mechanisms for resistance to occur can be expected to be life threatening to the mosquito. Despite the fact that TMOF expression increases *B.*

bassiana virulence to *A. gambiae*, LT50 values of *Bb*-Aa1 infected mosquitoes suggest that death is not quick enough to completely inhibit females from going through their gonotrophic cycles, even if spores were captured a couple of days before a blood feed. It has been proposed that insecticides exhibiting slow death rates that favor some reproductive success in female mosquitoes can suppress or at least reduce the emergence of resistance (Read, *et al.*, 2009). Further, since fungal pathogenesis is a multi-factorial process, where TMOF is not required, but acts to augment virulence, the likelihood of resistance is decreased due to the range of host processes targeted by the fungus, which would have to be overcome. Finally, expression of TMOF does not appear to increase the general virulence of *B. bassiana* to other insects, i.e. Lepidoptera, indicating target specificity (Fan, *et al.*, 2012). Ideally, TMOF expressing fungi should be incorporated as part of integrated pest management (IPM) programs that do not rely on a single approach for insect control but which utilize compatible and synergistic approaches.

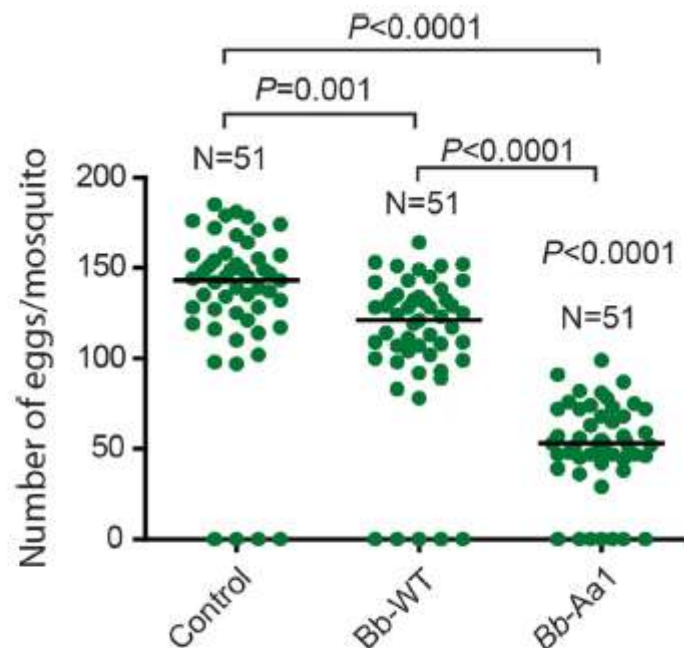


Figure 14. *Aea*-TMOF reduces fecundity of *Anopheles gambiae* mosquitoes. *A. gambiae* females sprayed with a suspension of 1×10^8 spores/ml of wild type or *Bb*-Aa1

strain, were given a blood meal 24 h after fungal infection. Non-infected and blood fed mosquitoes were used as controls. Each circle on the graph represents the number of eggs per mosquito. Medians are indicated by black lines and distributions were compared using the Kolmogorov-Smirnov test. N: number of mosquitoes in each group.

4. Conclusion

Our data show that expression of (*Aea*)-TMOF in a mycoinsecticide can increase its effectiveness against two important mosquito vectors, *Ae. aegypti* and *A. gambiae*. Both survival times and the median lethal dose were lower in the *Aea*-TMOF expressing strain as compared to the wild type parent. In addition, target fecundity was dramatically reduced. Additional research is needed to examine issues of persistence, delivery, and even greater targeting of hosts, however, the principle of exploiting critical target host molecules for expression in an insect pathogen, which, during infection would compromise the host, can be expanded to a wide range of applications in the biological control of insects.

B. The mosquito melanization response is implicated in defense against the entomopathogenic fungus *Beauveria bassiana*

1. Abstract

Mosquito immunity studies have focussed mainly on characterizing immune effector mechanisms elicited against parasites, bacteria and more recently, viruses. However, those elicited against entomopathogenic fungi remain poorly understood, despite the ubiquitous nature of these microorganisms and their unique invasion route that bypasses the midgut epithelium, an important immune tissue and physical barrier. Here, we used the malaria vector *Anopheles gambiae* as a model to investigate the role of melanization, a potent immune effector mechanism of arthropods, in mosquito defense against the entomopathogenic fungus *Beauveria bassiana*, using *in vivo* functional genetic analysis and confocal microscopy. The temporal monitoring of fungal growth in mosquitoes injected with *B. bassiana* conidia showed that melanin eventually formed on all stages, including conidia, germ tubes and hyphae, except the single cell hyphal bodies. Nevertheless, melanin rarely aborted the growth of any of these stages and the mycelium continued growing despite being melanized. Silencing *TEP1* and *CLIPA8*, key positive regulators of *Plasmodium* and bacterial melanization in *A. gambiae*, abolished completely melanin formation on hyphae but not on germinating conidia or germ tubes. The detection of a layer of hemocytes surrounding germinating conidia but not hyphae suggested that melanization of early fungal stages is cell-mediated while that of late stages is a humoral response dependent on TEP1 and CLIPA8. Microscopic analysis revealed specific association of TEP1 with surfaces of hyphae and the requirement of both, TEP1 and CLIPA8, for recruiting phenoloxidase to these surfaces. Finally, fungal proliferation was more rapid in *TEP1* and *CLIPA8* knockdown mosquitoes which exhibited increased sensitivity to natural *B. bassiana*

infections than controls. In sum, the mosquito melanization response retards significantly *B. bassiana* growth and dissemination, a finding that may be exploited to design transgenic fungi with more potent bio-control activities against mosquitoes.

2. Introduction

Melanization is an immediate immune response in arthropods leading to the physical encapsulation of pathogens in a dense melanin coat, and to the generation of toxic metabolites that can harm certain pathogens. It is also a prominent wound healing process manifested by the blackening of the wound area in arthropods. Melanization is triggered by pattern recognition receptors (PRRs) that upon binding pathogen associated molecular patterns (PAMPs) activate a cascade of serine proteases culminating in the proteolytic cleavage and conversion of the prophenoloxidase (PPO) zymogen into active phenoloxidase (PO), the rate limiting enzyme in melanogenesis (Cerenius, *et al*., 2008). The protease cascade acting upstream of PPO involves often a modular protease and several downstream clip-domain serine proteases (CLIPs) (Park, *et al*., 2007; Wang and Jiang, 2006). This cascade is under tight temporal regulation by serine protease inhibitors (SRPNs). In the dipterans *Drosophila* and *Anopheles gambiae*, the absence of *SPN43Ac*, also called *necrotic*, (Levashina, *et al*., 1999b) and SPRN2 (Michel, *et al*., 2005), respectively, resulted in the appearance of spontaneous melanotic pseudotumors in adult tissues and a reduced life span, suggesting that aberrant control of melanization imposes a fitness cost on the host. Further, this process is regulated spatially which ensures that melanin formation occurs exclusively on microbial surfaces minimizing collateral damage to the host.

Biochemical studies in *Manduca sexta* (Yu, *et al*., 2003) and *Tenebrio molitor* (Lee, *et al*., 2002) revealed that PPO activation is further controlled by the requirement

of non-catalytic CLIPs [also known as serine protease homologs (SPHs)] as co-factors for prophenoloxidase activating enzymes (PPAE) to trigger proper processing of PPO into PO. SPHs have substitutions at one or more of the critical His/Asp/Ser triad that renders them non-catalytic. Functional genetic studies in the malaria vector *A. gambiae* revealed that a clip domain-containing SPH termed CLIPA8 is required for the melanization of *Plasmodium berghei* ookinetes in the mosquito midgut (Volz, *et al.*, 2006) and bacteria in the hemocoel (Schnitger, *et al.*, 2007). While there is no evidence yet for the direct involvement of CLIPA8 in the processing of PPO, these studies provided a strong genetic evidence for the central role of SPHs in the melanization response *in vivo*.

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). Abolishing PO activity in the malaria vector *A. gambiae*, by silencing CLIPA8, did not affect mosquito survival after infections with *Escherichia coli* or *Staphylococcus aureus* (Schnitger, *et al.*, 2007). Both bacterial species were cleared from CLIPA8-silenced mosquitoes as efficiently as from controls suggesting that melanization is not critical for anti-bacterial defense in the mosquito. In *A. gambiae*, the melanization response to *P. berghei* is also controlled by CLIPA8 (Volz, *et al.*, 2006), in addition to the complement-like protein TEP1 (Blandin, 2004) and two leucine-rich immune proteins, LRIM1 (Osta, *et al.*, 2004) and APL1C (Riehle, *et al.*, 2006; Riehle, *et al.*, 2008). The latter two proteins form an obligate disulfide-linked heterodimer in the mosquito hemolymph that interacts with and stabilizes a cleaved form of TEP1 (Fraiture, *et al.*, 2009; Povelones, *et al.*, 2009). In addition to triggering ookinete lysis in the basal labyrinth of the midgut epithelium (Blandin, *et al.*, 2004; Osta, *et al.*, 2004; Riehle, *et al.*, 2006), the TEP1/LRIM1/APL1C complex (henceforth TEP1 complex) is also required for the melanotic response to ookinetes in refractory mosquito genotypes (Blandin, *et al.*, 2004; Osta, *et al.*, 2004) as well as to bacteria injected directly into the hemolymph (unpublished data). Nevertheless, wild-type laboratory and field caught *A. gambiae* mosquitoes rarely melanize malaria parasites (Niare, *et al.*, 2002) and melanization does not seem to be important for *A. gambiae* anti-bacterial defense (Schnitger, *et al.*, 2007), questioning the role of this response in mosquito immunity.

Research on mosquito immunity has focused mainly on parasites, bacteria (Reviewed in Yassine and Osta, 2010) and lately viruses (Sessions, *et al.*, 2009; Souza-Neto, *et al.*, 2009; Waldock, *et al.*, 2012), whereas entomopathogenic fungi received little attention despite their ubiquitous nature and their route of infection which unlike

other microbial classes does not require ingestion by the host. Rather, these fungi infect by direct penetration through the mosquito cuticle into the hemolymph. This mode of infection is particularly attractive for immunity studies because it does not require artificial injection of the microbe into the hemolymph. It also bypasses the midgut epithelium which was shown recently to be engaged in promoting complement-mediated ookinete lysis in the basal labyrinth of the midgut epithelium, by triggering intracellular nitration of ookinete surface proteins (Oliveira Gde, *et al*., 2012). Here, we investigate the role of melanization in defense against natural infections with the entomopathogenic fungus *B. bassiana* and provide novel insights into the cellular and molecular mechanisms triggering fungal melanization *in vivo*.

3. Results

a. *Beauveria bassiana* infection triggers the melanization response of *A. gambiae*

Mosquito immune responses to entomopathogenic fungi such as *B. bassiana* remain poorly understood. We carried a meticulous microscopic analysis of *B. bassiana* development in adult *A. gambiae* mosquitoes to determine whether melanization is also triggered in this model infection system and against which stages. To facilitate detection of the fungus in dissected mosquito abdomens we utilized a GFP-expressing strain of *B. bassiana* (Bidochka, *et al*., 2010). Individual mosquitoes were injected intrathoracically with 200 freshly prepared conidia (spores) and fungal development was monitored in dissected abdomens at 1, 6, 12, 24 and 48 h post-infection (pi). In these assays, mosquitoes were infected by injecting conidia rather than by the natural route (tarsal contact with spores), because in the former mycelial growth was frequently observed in dissected abdomens which was not the case in natural infections. Most conidia were rapidly melanized at 1 hr pi; these appeared black since GFP was masked by the

melanotic capsule (Figure 15A). Only rare non-melanized conidia were observed at that time point. At 6 h pi all conidia were melanized (Figure 15B), however, at later time points, some melanized conidia exhibited an enlarged size indicating that germination was taking place within the melanotic capsules (Figure 15C). Indeed, at 24 h pi, germ tubes started emerging from melanized conidia concomitant with melanin formation around their walls (Figure 15D). Two days pi, melanin was also detected on several hyphae that emerged from germ tubes, while few hyphae remained non-melanized (Figures 15E and 15F). Altogether, our data revealed that conidia, germ tubes and hyphae were efficiently melanized in *A. gambiae* mosquitoes, yet this immune reaction was not sufficient to abort completely the development of the mycelium. However, we noticed that hyphal bodies, yeast-like single-cells that differentiate from growing hyphae, were rare and sometimes absent in abdomens at 48 h post conidia injection, whereas these stages were commonly present in the hemolymph of other insect species at the same time point (Pendland, *et al*., 1993; Wanchoo, *et al*., 2009). This suggests that melanization might be retarding the growth of the fungus in the mosquito.

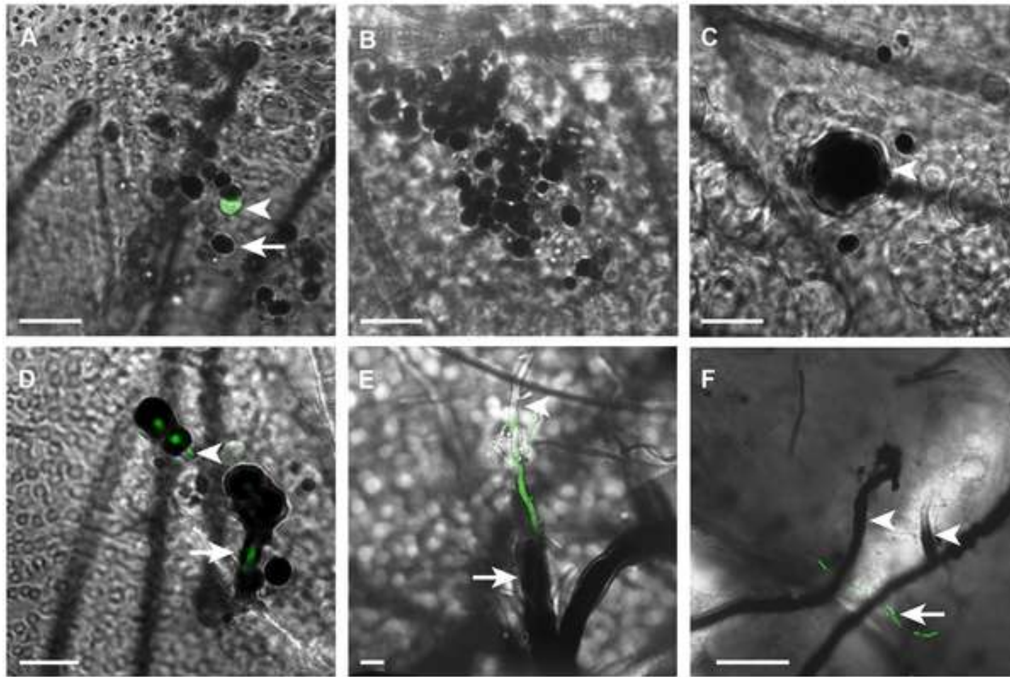


Figure 15. Mosquito melanization response to *B. bassiana* developmental stages. Merged fluorescent and bright field images of abdomens dissected from mosquitoes at (A) 1 hr, (B) 6 h, (C) 12 h, (D) 24 h and (E and F) 48 h following the injection of each with 200 conidia of GFP-expressing *B. bassiana*. (A) Conidia were rapidly melanized (arrow) 1 h post-injection (pi); few non-melanized conidia were detected at that time point (arrowhead). (B) All conidia were melanized at 6 h pi. (C) An enlarged conidium germinating within the melanotic capsule (arrowhead) at 12 h pi. (D) A germ tube breaking through the melanin coat at 24 h pi (arrowhead) and another elongating with concomitant melanin formation around its wall (arrow). (E) Partially melanized hyphae in a mycelium at 48 h pi showing absence of melanin at the apical part (arrowhead) and presence of a thick melanin coat around the basal part (arrow). (F) A low magnification image showing extensive melanization of hyphae (arrowheads) in the growing mycelium at 48 h pi, with few GFP-expressing, non-melanized hyphae detected (arrow). Scale bars are 10 μm in A-E and 50 μm in F.

a. Cellular and humoral melanotic responses elicited against *B. bassiana* in adult mosquitoes

The melanization of *P. berghei* in certain refractory *A. gambiae* mosquito genotypes is a humoral response dependent on CLIPA8 (Volz, *et al*., 2006), and TEP1 complex (Blandin, *et al*., 2004; Fraiture, *et al*., 2009; Osta, *et al*., 2004; Povelones, *et al*., 2009). In this model system, the midgut basal lamina constitutes a physical barrier that inhibits direct contact between hemocytes and ookinetes residing in the basal labyrinth. Bacteria injected into the hemolymph also elicit a humoral melanotic

response dependent on CLIPA8 (Schnitger, *et al*., 2007) and TEP1 complex (unpublished data), suggesting that these are core proteins in the mosquito melanization response. To address whether they exhibit similar roles in infections with *B. bassiana*, *TEP1* and *CLIPA8* were silenced in adult female mosquitoes by RNAi knockdown (kd) (Blandin, *et al*., 2002), then individual mosquitoes were injected with 200 conidia of GFP-expressing *B. bassiana*. Mosquito abdomens were dissected two days after spore injection in order to score fungal melanization. Western blot analysis of hemolymph extracts confirmed that *TEP1* and *CLIPA8* were efficiently silenced four days after injection of their corresponding double-stranded RNAs (Figure 16A). In *LacZ* kd control mosquitoes, a thick melanin coat covered the majority of the growing mycelium as expected (Figure 16B). In contrast, hyphal melanization was completely abolished in *CLIPA8* and *TEP1* kd mosquitoes, suggesting that these proteins are indeed core regulators of the melanization response (Figures 16C and 16D, respectively). Intriguingly, in these two genotypes, only the base of the growing mycelium from which hyphae emerged was still melanized as efficiently as in *LacZ* kd controls. These findings were unexpected since the same gene knockdowns completely abolished the melanotic response to *P. berghei* (Blandin, *et al*., 2004; Volz, *et al*., 2006) and bacterial infections (Schnitger, *et al*., 2007; and unpublished data).

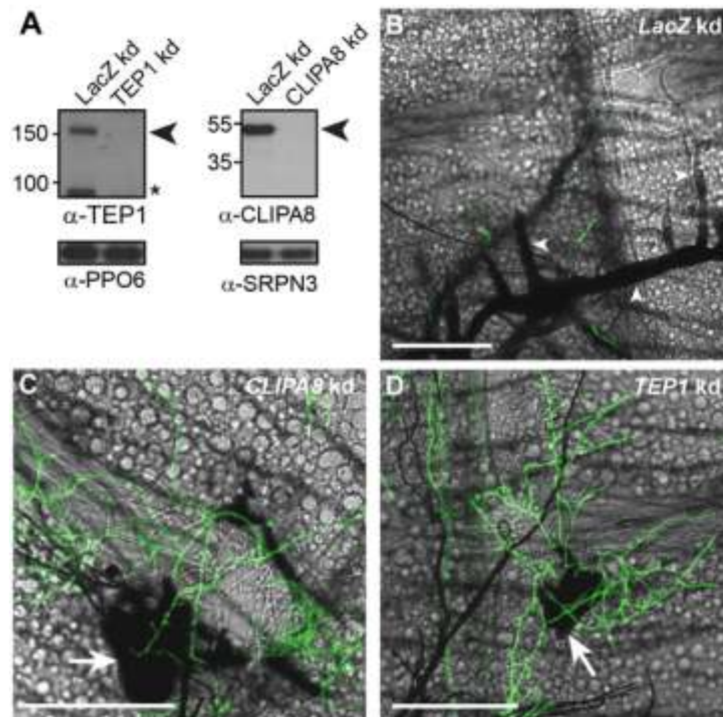


Figure 16. TEP1 and CLIPA8 are required for the melanization of hyphae. (A) Western blot analysis showing CLIPA8 and TEP1 depletion in the hemolymph four days after silencing their corresponding genes by RNAi. *Left*, arrowhead indicates full-length TEP1 (TEP1-F) and asterisk denote the cleaved form of TEP1 (TEP1-C); *Right*, arrowhead indicates full length CLIPA8. PPO6 and SRPN3 were used as loading controls. (B) Abdomens dissected from *LacZ* kd mosquitoes at 48 h post-injection of 200 conidia of GFP-expressing *B. bassiana* (Green), show intense melanization of hyphae (arrowheads). (C and D) Absence of melanin around hyphae in *CLIPA8* and *TEP1* kd mosquitoes, respectively. Note, however, the strong melanization associated with the base of the mycelium in each of these genotypes (arrows). Scale bars are 100 μm.

We hypothesized that two distinct mechanisms are driving the melanotic response to the fungus. The first is hemocyte-mediated and targets the early stages of fungal development in the mosquito, including the germinating spores and germ tubes. The second is humoral, dependent on TEP1 and CLIPA8 functions, and targets the hyphae that develop later. To address this point, abdomens dissected from wild-type mosquitoes, at 12 and 48 h after conidia injection were immunostained with polyclonal antibody against PPO6, which is known to be expressed in hemocytes (Muller, *et al.*, 1999; Pinto, *et al.*, 2009). Abdomens dissected at the earlier time point clearly showed a

circular arrangement of hemocytes around enlarged conidia that were apparently germinating within the melanotic capsule (Figure 17A). Most of these hemocytes showed absence of, or a faint PPO signal possibly because they have exhausted their PPO content in the struggle against the germinating conidium. Alternatively, some of these hemocyte may not express PPO. A hemocyte strongly expressing PPO was resting on top of two other hemocytes that are in direct contact with the conidium (Figure 17A), suggesting that hemocytes recruited to the germinating spore may form more than one layer around it attempting to abort its growth, pretty much similar to nodule formation in larger insects. At 48 h after infection, no hemocytes were detected in close proximity to melanized hyphae supporting the humoral nature of this response (Figure 17B). The hyphal tips from where growth occurs exhibited a thin, often barely detectable layer of melanin, but a strong PPO signal (Figure 17B), suggesting that melanin biosynthetic reactions were still particularly active at these foci. Yet, the whole process was taking place in the absence of hemocytes from the hyphal tips.

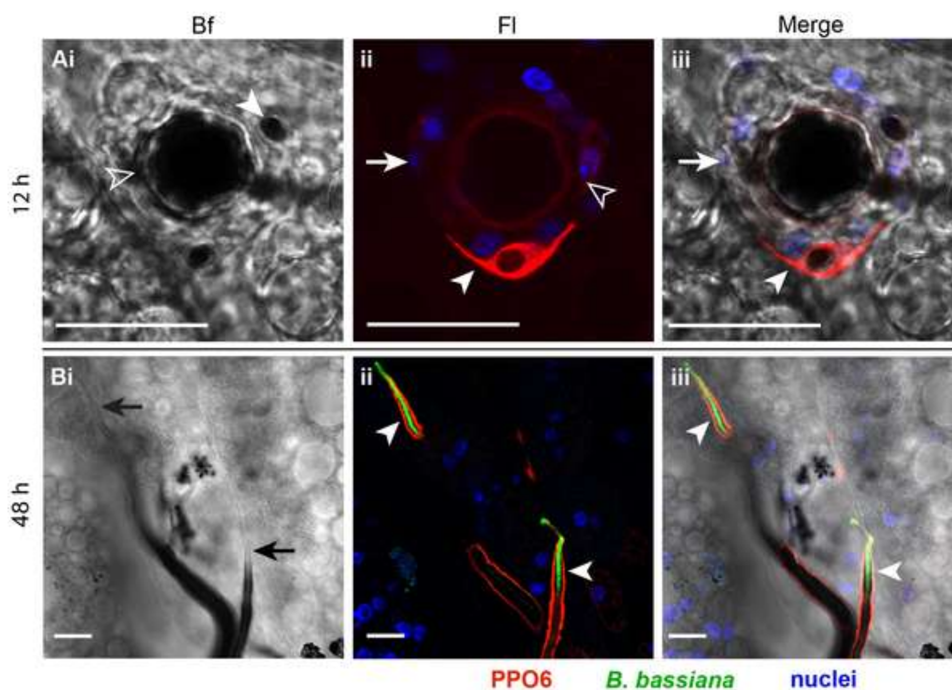


Figure 17. *B. bassiana* infection triggered cellular and humoral melanotic responses in the mosquito. Abdomens were dissected at (A) 12 and (B) 48 h post-

injection of each mosquito with 200 conidia of GFP-expressing *B. bassiana* and immunostained with α-PPO6 antibody. Shown are bright field (Bf), fluorescence (Fl), and merged bright field and fluorescence (Merge) images of confocal sections. (A) PPO staining of a germinating conidium. A conidium germinating despite being melanized (Ai, open arrowhead) exhibited an enlarged size compared to a nearby non-germinating spore (Ai, filled arrowhead). A cellular layer of hemocytes surrounds the germinating conidium (Aii and Aiii, arrows). These hemocytes exhibited no or faint PPO signal (Aii, open arrowhead). A hemocyte expressing PPO (Aii and Aiii, filled arrowhead) was resting on top of the cells that surrounded the germinating conidium. (B) PPO staining of hyphae. The apical parts of hyphal filaments exhibited a thin melanin coat (Bi, arrows) but strong PPO signal (Bii and Biii, arrowheads). The absence of hemocytes around these hyphae informs a humoral melanotic response. PPO6 (red), GFP-expressing *B. bassiana* (green), nuclei (blue). All scale bars are 20 μm.

The microscopic analysis described above indicates that the early melanotic response to conidia injection requires the direct participation of hemocytes while that triggered against growing hyphae is humoral and dependent on TEP1 and CLIPA8. To investigate further this point, we measured the temporal dynamics of hemolymph PO activity in *CLIPA8, TEP1* and *LacZ* kd mosquitoes at 24, 48 and 72 h after spraying with a suspension of 1 x 10^8 conidia/ml. PO activities in both *CLIPA8* (Figure 18A) and *TEP1* kd mosquitoes (Figure 18B) were similar to that in *LacZ* kd controls at 24 h post-challenge, when the fungus has just invaded the cuticle. However, at later time points, the activity dropped significantly in both *CLIPA8* and *TEP1* kd mosquitoes while it remained relatively unchanged in controls, which further supports the humoral nature of the melanotic response to late fungal stages.

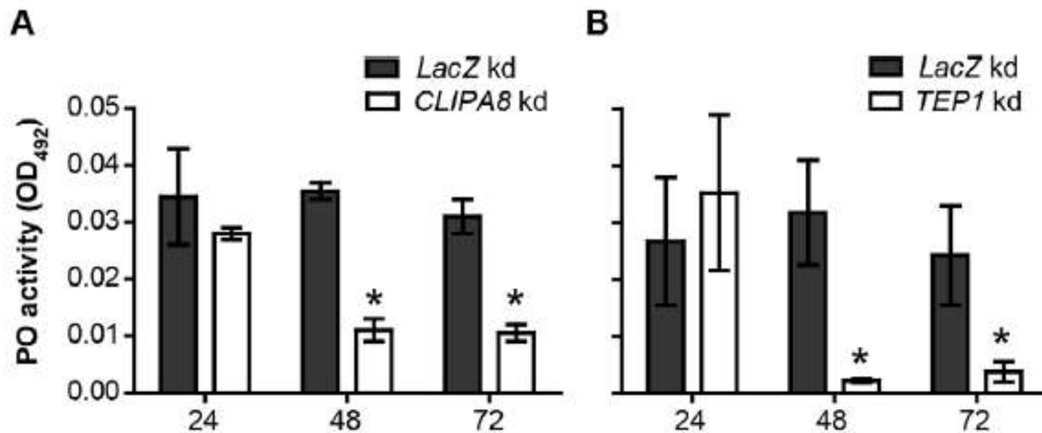


Figure 18. *CLIPA8* and *TEP1* kd abolished PO activity triggered during late but not early fungal infection. Phenoloxidase (PO) enzymatic activity [detected as absorbance at 492 nm, OD_{492}, after conversion of L-3,4-dihydroxyphenylalanine (L-DOPA)], was measured in hemolymph extracted from (A) *CLIPA8* and (B) *TEP1* kd mosquitoes and compared to that in *LacZ* kd controls at the indicated time points, after challenging mosquitoes by spraying with a *B. bassiana* (strain 80.2) suspension containing 1×10^8 conidia/ml. The graphs show PO activity measured at 50 min after addition of L-DOPA. Means were calculated from two independent biological experiments performed using different batches of mosquitoes and fungal conidia. Error bars represent the standard error of the mean. Statistical analysis was performed using the Student's t-test and differences were considered to be statistically significant if $P < 0.05$ (indicated by an asterisk).

c. PPO recruitment to hyphae is dependent on both TEP1 and CLIPA8

Initiation of the melanization reaction requires limited proteolytic cleavage of zymogen PPO into active PO, the rate limiting enzyme in melanogenesis. The mechanisms which trigger PPO recruitment to microbial surfaces remain unclear. Here, we analyzed PPO localization to hyphae in *TEP1*, *CLIPA8* and *LacZ* kd (control) mosquitoes at 48 h after conidia injection, using confocal microscopy. In the control group, PPO staining was observed on mycelial structures coated with a thick melanin capsule (data not shown) as previously reported in Figure 17B. Additionally, PPO was also detected along the length of hyphae on which melanin deposition was barely detectable or even absent (Figure 19A), as if a lag phase existed between PPO

recruitment and melanogenesis on these hyphal surfaces. PPO staining was often detected around the branching points of established hyphae (Figure 19A). Interestingly, silencing *CLIPA8* or *TEP1* completely abolished PPO localization to hyphae, and consequently none of these structures was melanized (Figures 19B and 19C, respectively). However, in these genotypes, PO was still detected on the melanized base of the mycelium from which hyphae emerged, corroborating our previous conclusion that the melanotic response against the early fungal stages is independent of TEP1 and CLIPA8 functions. Interestingly, the rare hyphal bodies detected in control mosquitoes at that time point, were not labelled with PPO (Figure 19A) suggesting that these stages might escape melanization.

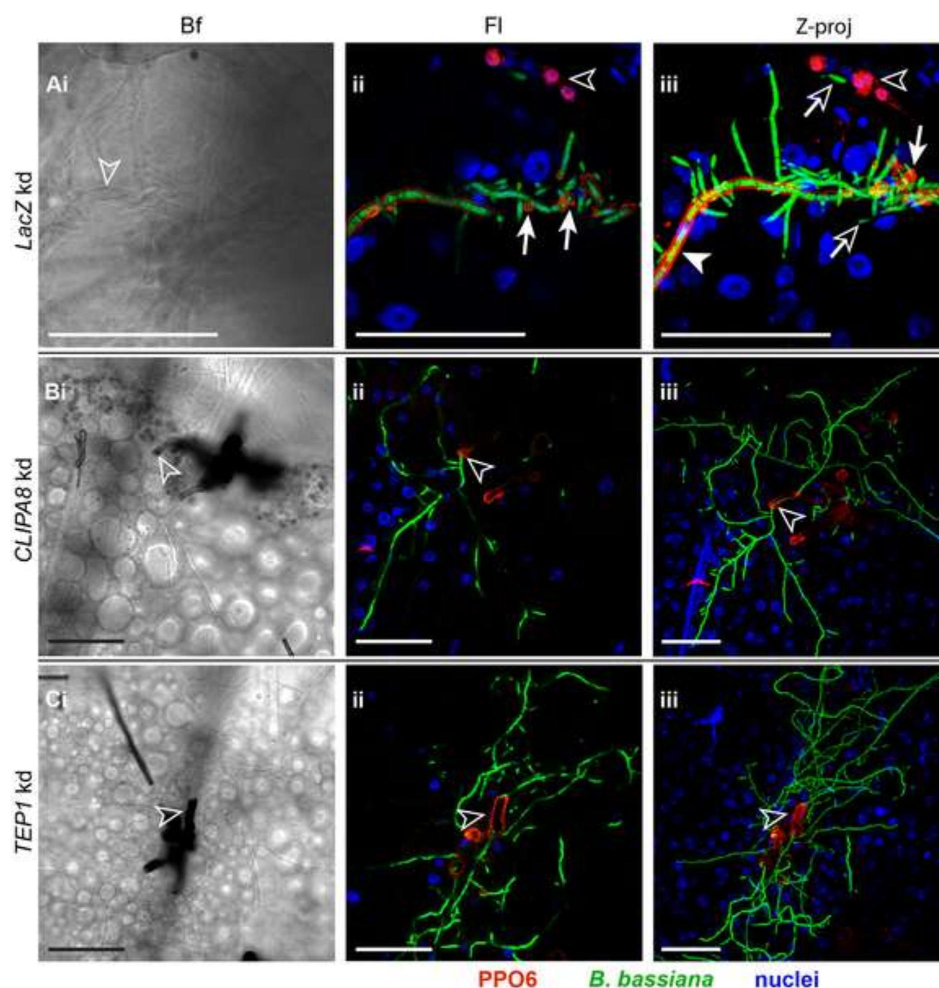


Figure 19. PPO recruitment to hyphae requires TEP1 and CLIPA8. Mosquito abdomens were dissected from (A) *LacZ*, (B) *CLIPA8* and (C) *TEP1* kd mosquitoes at

48 h post-injection of each with 200 conidia of GFP-expressing *B. bassiana* and stained with PPO6 antibody. Shown are Bright field (Bf) and fluorescence (Fl) images of confocal sections, and Z projections (Z-proj) of whole stacks. (A) An abdomen from *LacZ* kd mosquitoes at 100x magnification showed uniform PPO staining along an established hypha (Aiii, filled arrowhead) elaborating new hyphae and hyphal bodies. Note that melanin was barely detectable on this hyphal surface (Ai, open arrowhead) despite its intense PPO staining. A strong PPO signal was also observed at the branching points of the established hypha (Aii and Aiii, arrows with filled heads). The hyphal bodies detected were not labelled with PPO (Aii and Aiii, arrows with open heads). Shown also are PPO-expressing hemocytes (Aii and Aiii, open arrowheads). (B) *CLIPA8* and (C) *TEP1* kd mosquito abdomens at 40x magnification showing the absence of PPO and hence melanin from hyphal surfaces despite extensive mycelial growth in these genotypes; melanin formation (Bi and Ci, open arrowheads) and PPO staining (Bii-iii and Cii-iii, open arrowheads) were restricted only to the base of the mycelium from which hyphae emerged. PPO6 (red), *B. bassiana*-GFP (green) and nuclei (blue). All scale bars are 50 μm.

TEP1 binds to bacteria enhancing their phagocytosis by a hemocyte-like cell line (Levashina, *et al.*, 2001) and to *Plasmodium* ookinetes, as they egress from midgut epithelial cells into the basal labyrinth, leading to their lysis. The fact that TEP1 binds to evolutionary distant microbial surfaces and that PPO localization to hyphae is TEP1-dependent, prompted us to study whether TEP1 associates with hyphal surfaces to trigger downstream events culminating in PPO activation and subsequent melanin formation. Abdomens dissected from control (*LacZ* kd) mosquitoes at 48 h after conidia injections revealed strong TEP1 localization on melanin-free hyphal surfaces (Figure 20A); where melanin had previously formed, TEP1 signal was either faint or absent, possibly because it was masked by the thick melanotic capsule. Also, the rare hyphal bodies detected in these abdomens were labelled with TEP1. Thus, TEP1 localization to hyphal surfaces clearly precedes melanin formation; the tips from where hyphae grew were always TEP1 positive but melanin negative (Figure 20A). In *TEP1* kd mosquitoes, the melanization of hyphae was completely abolished (Figure 20B). Interestingly, hyphal bodies were more common in these mosquitoes relative to controls, suggesting rapid fungal growth. In summary, our data revealed that TEP1 association with hyphal

surfaces is a prerequisite for the initiation of a local melanotic reaction against *B. bassiana*.

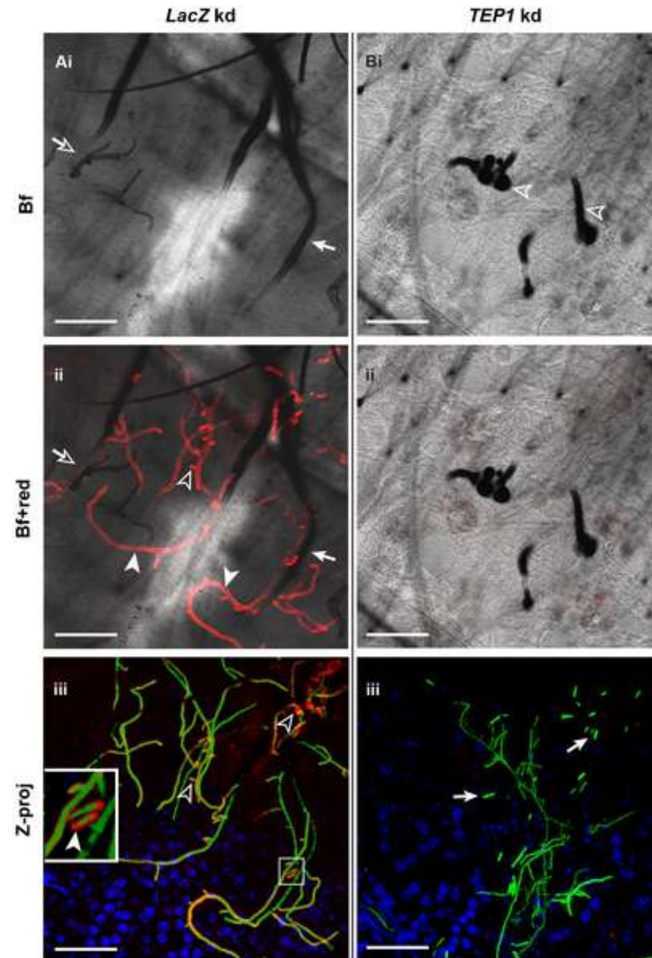


Figure 20. TEP1 localizes to hyphae and hyphal bodies. (A) *LacZ* (control) and (B) *TEP1* kd mosquitoes were injected each with 200 conidia of GFP-expressing *B. bassiana*. Abdomens were dissected 48 h later and immunostained with TEP1 antibody. Shown are bright field (Bf) and merged bright field and red channel (Bf+red) confocal sections and Z projections (Z-proj) of whole stacks. (A) In control mosquitoes TEP1 staining was observed on established hyphae (Aii, filled arrowheads), young branching hyphae (Aii and Aiii, open arrowheads) and hyphal bodies (Aiii, inset and filled arrowhead). Most TEP1-stained hyphae did not exhibit melanin formation at that time point (compare Ai and Aii), except in rare cases where TEP1 signal overlapped with a thin layer of melanin (Ai and Aii, arrows with open heads). TEP1 was not detected on heavily melanized hyphal surfaces probably because it was masked by melanin (Ai and Aii, arrows with filled heads). (B) TEP1 staining of hyphae was completely abolished in *TEP1* kd mosquitoes (compare Aii with Bii, and Aiii with Biii). Consequently, melanin did not form over these hyphal surfaces but was present only on early fungal stages including germinating spores and germ tubes (Bi, open arrowheads). Note the abundance of hyphal bodies in *TEP1* kd (Biii, arrows) compared to controls. TEP1 (red) GFP-expressing *B. bassiana* (green), nuclei (blue). All scale bars are 50 µm.

d. The mosquito melanization response protects against *B. bassiana* infection

The microscopic observation of melanotic capsules around entomopathogenic fungi has been reported earlier in several insect species including *Chironomus* (Gotz, *et al.*,1987), the leafhopper *Empoasca fabae* (Butt, *et al*., 1988) and the grasshopper *Melanoplus sanguinipes* (Bidochka and Khachatourians, 1987). However, the relative contribution of this immune response to anti-fungal defense remains poorly understood. In *A. gambiae*, melanization is dispensable for defense against bacterial infections, despite the fact that bacteria trigger PPO activation in the hemolymph (Schnitger, *et al*., 2007). Additionally, field caught *A. gambiae* mosquitoes (Niare, *et al*., 2002) as well as most laboratory strains rarely melanize *Plasmodium* ookinetes suggesting that melanization is dispensable for defense against these parasite stages. The fact that mosquitoes mounted a strong melanotic response to *B. bassiana* prompted us to test the relevance of this response to anti-fungal immunity. To address this point, *TEP1*, *CLIPA8* and *LacZ* kd adult female mosquitoes were naturally infected with a wild-type *B. bassiana* strain (80.2) either by spraying with a suspension of 1 x 10^8 condia/ml or by gentle dragging over a lawn of spores on a potato dextrose agar plate. Mosquitoes were then incubated at 27°C at 90% humidity and their survival was scored on a daily basis. Survival assays revealed a significant increase in susceptibility of *CLIPA8* and *TEP1* kd mosquitoes to *B. bassiana* over controls, whether by gentle dragging (Figure 21A) or spraying (Figure 21B) that were used to establish an infection. Interestingly, the *TEP1* kd group succumbed more quickly to infection than the *CLIPA8* kd, suggesting that TEP1 might be controlling more than one anti-fungal effector mechanism.

We then scored hyphal body colony forming units in *CLIPA8, TEP1* and *LacZ* kd mosquitoes four days after spraying with 5 x 10^7 conidia/ml, to determine whether the compromised survival in the two former genotypes is due to increased fungal

proliferation. Our data revealed that *CLIPA8* and *TEP1* kd mosquitoes contained indeed significantly higher numbers of hyphal bodies than controls which contained none at that time point; the median values were 15, 50 and 0, respectively (Figure 21C). Here, it is worth mentioning that, in general, hyphal bodies appeared more quickly in wild-type mosquitoes when fungal infection was established through injection rather than the natural route. This explains why these stages were sometimes detected in whole mounts of abdomens at 48 h post conidia injection (Figures 19A and 19B), but were absent from mosquitoes even at day four after natural infection (Figure 21C). Hyphal bodies were significantly more abundant in *TEP1* (*P*=0.0017) than in *CLIPA8* kd mosquitoes, which explains the increased sensitivity of the former genotype to *B. bassiana* challenge.

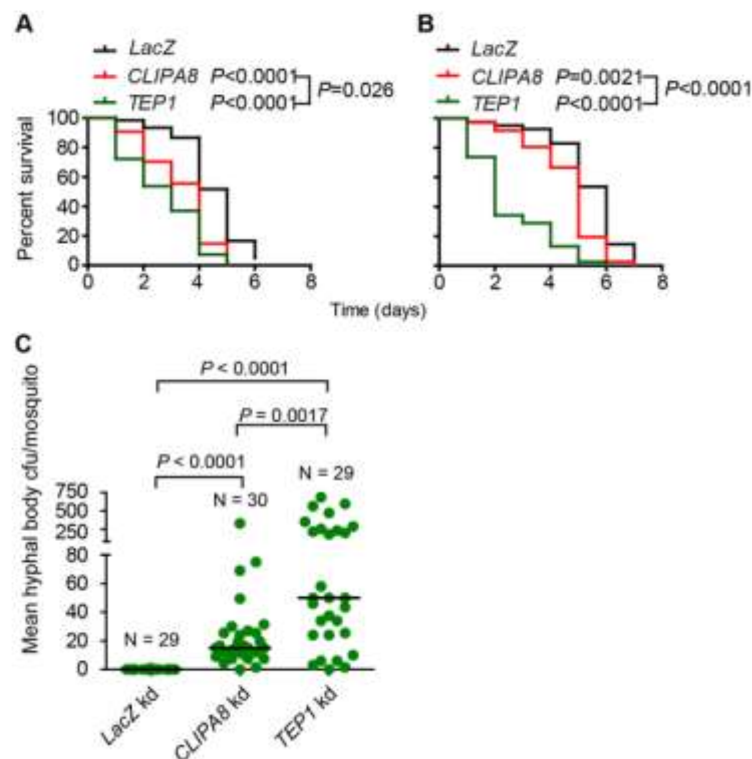


Figure 21. *CLIPA8* and *TEP1* kd mosquitoes are more sensitive to *B. bassiana* infections. *LacZ*, *TEP1* and *CLIPA8* kd adult female *A. gambiae* mosquitoes were challenged with *B. bassiana* (strain 80.2) either by (A) bringing the mosquitoes in contact with a lawn of conidia on fungal PDA plates or (B) by spraying mosquitoes with a suspension of 1 x 10^8 conidia/ml. Dead mosquitoes were counted daily over the indicated period. Graphs represent percent survival as calculated by the Kaplan-Meier

method for one representative experiment of each treatment. Statistical significance was calculated by the log rank test. Survival curves were considered to be significantly different if $P<0.05$. (C) *B. bassiana* infected *CLIPA8* and *TEP1* kd mosquitoes contained significantly more hyphal body colony forming units (cfu) than infected *LacZ* kd controls. Here, mosquitoes were challenged by spraying with *B. bassiana* strain 80.2 at a suspension of 5 x 10^7 conidia/ml and batches of two mosquitoes each were collected four days later, surface sterilized, grinded and serial dilutions plated on *B. bassiana* selective medium. Each circle represents mean hyphal body cfu per mosquito per batch. Medians are indicated with horizontal lines and were 0, 15 and 50 for *LacZ*, *CLIPA8* and *TEP1* kd mosquitoes, respectively. The numbers of batches (N) processed per genotype are indicated. Statistical significance was calculated using the Mann-Whitney test; means were considered significant if $P<0.05$. Results are from two independent biological experiments involving different batches of mosquitoes and fungal conidia.

4. Discussion

Melanization is an important immune response in insects that is triggered against diverse microbial classes including parasites (Li, *et al*., 1992), bacteria (Ayres and Schneider, 2008; Hillyer, *et al*., 2004; Schnitger, *et al*., 2007) and fungi (Bidochka and Khachatourians, 1987; Butt, *et al*., 1988; Gotz, *et al*., 1987). Functional genetic studies in several insect species revealed an important role for this response in insect immunity to bacterial infections (Ayres and Schneider, 2008; Eleftherianos, *et al*., 2007; Liu, *et al*., 2007). Here, we investigated the role of melanization in *A. gambiae* anti-fungal defense using *B. bassiana* as a model. Our work has been prompted by early electron microscopy studies showing the formation of melanotic capsules around pathogenic fungi invading the hemocoel of other insect species (Bidochka and Khachatourians, 1987; Butt, *et al*., 1988), and by the fact that entomopathogenic fungi employ a different route for mosquito invasion compared to bacteria and *Plasmodium* parasites. The latter two naturally infect through the oral route and traverse the midgut epithelium in order to gain access into the hemocoel, whereas pathogenic fungi breach the cuticle reaching directly into the hemocoel using a combination of mechanical pressure and an array of cuticle-degrading enzymes (St. Leger, *et al*.,1986).

Results obtained from temporal analysis of the melanotic response to *B. bassiana* developmental stages in adult mosquitoes, using fluorescent microscopy, are in line with early reports showing that this response did not prevent the germination of *B. bassiana* conidia in the hemolymph of other insect species (Bidochka and Khachatourians, 1987; Gotz and Vey, 1974). Melanization occurred rapidly on injected conidia, then progressed over the germ tubes as well as hyphae that constitute the bulk of the mycelium (Figure 15). Only in rare cases was the mycelium completely melanized, rather hyphae almost always succeeded to break through the melanotic capsule. Our results revealed that the mosquito mounts a potent melanotic response against the fungus, with melanized hyphae sometimes measuring more than one millimeter in length (data not shown). This response, however, is not sufficient to kill the fungus. A possible explanation could be the depletion of hemolymph PPO later during infection, due to the continuous triggering of the response by the rapidly growing fungus; however, western blot analysis excluded such possibility since PPO levels remained relatively unchanged in the hemolymph up to five days post-infection (Figure 22). Nevertheless, we provided, for the first time, tangible evidence that melanization retards significantly the growth of the fungus in the mosquito. This is reflected in the absence of hyphal bodies in control (*LacZ* kd) mosquitoes four days after spraying with a conidial suspension, compared to their presence in *CLIPA8* and *TEP1* kd mosquitoes processed at the same time (Figure 21C).

The delay in the differentiation of hyphal bodies in control mosquitoes is probably imposed by the strong melanotic response triggered against hyphae. This is supported by the detection of PPO and TEP1 staining not only on hyphae but also around the branching points where new hyphae and possibly hyphal bodies emerged (Figures 19A and 19A, respectively). Delaying or inhibiting hyphal body differentiation

may limit fungal dissemination, since these single cell stages proliferate in the hemolymph ultimately establishing their own mycelia. It was previously reported that melanin exhibits anti-fungal properties *in vitro* against *Aphanomyces astaci* (Soderhall and Ajaxon, 1982) and *Metarhizium anisopliae* (St. Leger, *et al.,* 1988); however, the mechanism by which it interferes with fungal growth is still not clear. A plausible explanation is the ability of melanin to bind and inhibit the activity of a wide range of proteins (Doering, *et al*., 1999) including lytic enzymes produced by microbes, such as chitinases, which are involved in fungal cell wall remodelling during cell division (Kuo and Alexander, 1967). Hence, melanin might slow down fungal growth by interfering with the synthesis of new cell wall material during that process.

Figure 22. Hemolymph PPO levels after natural *B. bassiana* infection. Western blot analysis showing hemolymph PPO levels in adult female mosquitoes at the indicated times points after spraying them with a suspension of 1 x 10^8 conidia/ml of *B. bassiana* (strain 80.2). Each lane contains hemolymph extracts from 20 mosquitoes. C: control non-infected mosquitoes. Asterisks indicate non-specific bands.

The rare hyphal bodies detected in control mosquitoes at 48 h after conidia injection were not melanized nor exhibited a PPO signal (Figure 19A), suggesting that they escape melanization. The evasion of host defense by these *in vivo* stages have been proposed earlier and was attributed to their minimal cell wall which lacks immuno-stimulatory carbohydrates (Pendland, *et al*.,1993). A more recent study based on lectin-

mapping revealed that *B. bassiana* developmental stages exhibit differences in the composition of surface carbohydrates, in particular hyphal bodies which seem to shed most carbohydrate epitopes from their surface (Wanchoo, *et al.*, 2009). This minimal cell wall, however, did not prevent TEP1 association with the surface of hyphal bodies (Figure 20A). TEP1 recruitment to GFP-expressing *P. berghei* ookinetes triggers parasite lysis as reflected by the loss of cytoplasmic GFP signal and membrane blebbing (Blandin, *et al*., 2004). TEP1 labelled hyphal bodies were still expressing GFP and did not show an aberrant morphology, however, it is difficult to conclude at that stage whether they are live or not without detailed electron microscopy analysis. Nevertheless, the fact *TEP1* kd mosquitoes exhibited significantly higher numbers of hyphal bodies and increased sensitivity to natural *B. bassiana* infections compared to *CLIPA8* kd, inform an additional, melanization-independent role of TEP1 in limiting fungal growth, that remains to be defined. TEP1 is known to be an important anti-bacterial factor (Dong, *et al*., 2006a; Levashina, *et al.*, 2001), which raises the possibility that the rapid death observed in fungal infected *TEP1* kd mosquitoes could be due to the proliferation of opportunistic bacterial infections rather than fungal proliferation. However, this possibility was excluded because fungal infection triggered a similar survival pattern in *TEP1* kd mosquitoes pre-treated with a cocktail of antibiotics (Figure 23).

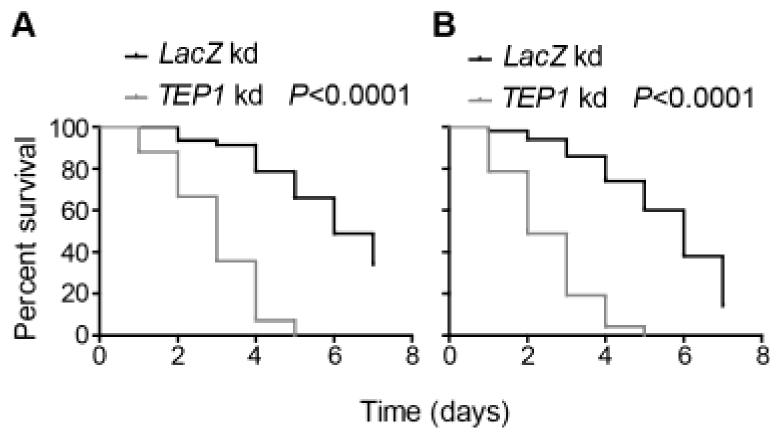


Figure 23. *TEP1*-silenced aseptic mosquitoes are still sensitive to *B. bassiana* infection. *LacZ* and *TEP1* kd female *A. gambiae* mosquitoes treated with antibiotics, to eliminate or at least reduce substantially their microbial flora, were challenged with *B. bassiana* (strain 80.2) by spraying mosquitoes with a suspension of 1 x 10^8 conidia/ml. Dead mosquitoes were counted daily over the indicated period. (A) and (B) Two independent experiments performed with different batches of mosquitoes and fungal conidia. Graphs represent percent survival as calculated by the Kaplan-Meier method. Statistical significance was calculated by the log rank test. Survival curves were considered to be significantly different if $P<0.05$.

Using immunohistochemistry and confocal microscopy, we observed a PPO-positive signal on hyphae that exhibited either minimal or no melanin formation (at least within the resolving power of light microscopy) in control mosquitoes (Figure 19A). On these hyphae melanogenesis appeared lagging behind fungal growth, whereby apical parts of hyphae exhibited strong PPO staining but minimal or no melanin formation, while the basal parts showed PPO staining around thick melanotic capsules. This is the first time mosquito PPO is detected on microbial surfaces that do not exhibit clear signs of melanin formation. In *A. gambiae-P.berghei* model system, a PPO signal was always detected around dead parasites confined in a dense melanotic capsule (Blandin and Levashina, 2004a). A plausible explanation for this unusual pattern of PPO localization to hyphae is that the rapidly growing fungus probably exhausts the mosquito melanotic response. This is supported by the finding that PO activity in control mosquitoes did not

change significantly between 24 and 72 h post infection, suggesting continuous PPO activation, at least during that period (Figure 18).

The depletion of TEP1 or CLIPA8 from mosquito hemolymph completely abolished PPO localization to hyphae and their subsequent melanization, suggesting that PPO recruitment to fungal surfaces is an indirect process that depends, most likely, on the prior assembly of an immune protein complex on microbial surfaces. Whether this complex includes TEP1 and CLIPA8, and whether these two proteins recruit PPO directly or indirectly to microbial surfaces remain to be elucidated. Our data also revealed that, in addition to their role as cofactors for PPO activation (Piao, *et al.*, 2005; Yu, *et al.*, 2003), clip-domain serine protease homologs seem to be required for PPO recruitment to microbial surfaces. It was previously reported that TEP1 binding to ookinetes in a melanotic refractory strain of *A. gambiae*, triggered their lysis and subsequent melanotic encapsulation. In that study, the authors proposed that PPO activation and recruitment was triggered by dead parasites, already killed by TEP1, and not by TEP1 itself, suggesting that the melanotic response is reminiscent of wound healing and does not represent an immune defense reaction *per se* (Blandin, *et al.*, 2004). It is difficult to reconcile our findings with those of the above study for the following reasons. First, in our infection model, PPO does not seem to be recruited to the surface of a dying fungus since melanized hyphae were not killed and were still growing at their tips, often elaborating lateral branches. Second, even though we did not assay directly the co-localization of TEP1 and PO (both antibodies were produced in the same host species), the fact that both were able to bind hyphae exhibiting minimal or no melanin formation, suggests that they might co-localize on hyphal surfaces.

The *A. gambiae* melanization response can be triggered by bacteria (Schnitger, *et al*., 2007), *Plasmodium* ookinetes (Collins, *et al*., 1986; Osta, *et al*., 2004), Sephadex

beads (Warr, *et al* ., 2006) and fungi (according to this report). Further, certain immune proteins like TEP1 (Blandin, *et al* ., 2004; Warr, *et al*., 2006; unpublished data) and CLIPA8 (Schnitger, *et al*., 2007; Volz, *et al*., 2006) are required in all these melanotic events; of note, the role of CLIPA8 in bead melanization has not been addressed but is expected to be also essential. Altogether, these findings indicate that the molecular mechanisms that underlie the mosquito melanization response to foreign bodies with distinct biochemical surface characteristics are controlled to a certain extent by the same genetic loci. They also raise intriguing questions as to the nature of the upstream molecular recognition process that triggers the melanotic response to each of these foreign surfaces, especially that Sephadex beads, which are inanimate bodies, are still efficiently melanized in the hemolymph.

In this report, we also showed that the mosquito elicits both cellular and humoral melanotic responses against *B. bassiana*. The fact that neither *TEP1* nor *CLIPA8* kd abolished melanization of the early fungal stages (Figures 16 and 20B) despite being essential proteins in this response (Blandin, *et al*., 2004; Schnitger, *et al*., 2007; Volz, *et al*., 2006), and the detection of a layer of hemocytes around germinating spores (Figure 17A), suggested an early cellular melanotic response. These findings challenge the current belief that melanization in insect stages with limited numbers of hemocytes, such as adult mosquitoes, occurs in a humoral manner without the direct participation of hemocytes (Goetz, *et al*.,1977). It is possible that these cellular responses were missed because they are rare events elicited in specific cases against particular pathogens. Surprisingly, no hemocytes were observed close to hyphae later during infection, indicating that the melanotic response to these stages is humoral. There are two plausible explanations for this phenomenon which are not necessarily mutually exclusive. First, lectin mapping assays revealed that different developmental

stages of *B. bassiana* display diverse surface carbohydrates (Wanchoo, *et al*.,2009). Since sugars play important roles in non-self recognition, distinct sugar signatures may elicit different immune responses. Second, *B. bassiana* may interfere, at some point during its development, with the migration of hemocytes, as previously reported in the larvae of *Spodoptera exigua* infected with this fungus (Hung, *et al*.,1993). We would like to point out however, that the melanotic response elicited against hyphae, although humoral in nature, still depends on an indirect role of hemocytes being the main producers of many immunity proteins including TEP1 (Levashina, *et al*., 2001) PPO and CLIPA8 (Pinto, *et al*., 2009).

In summary, the interactions between the mosquito melanotic response and *B. bassiana* are evocative of an "arms race" where the fungus is almost always the winner. This does not mean that melanization is not protective; we have provided evidence that this immune response retards significantly fungal growth, and might severely compromise or even completely abrogate the growth of fungi that are less virulent than *B. bassiana*. In fact, by successfully adapting to a particularly wide host range, *B. bassiana* must have evolved strategies to overcome insect immune defenses and enhance its pathogenesis. This is supported by recent insights from the *B. bassiana* genome which revealed species-specific expansions of gene families encoding toxins, proteases and putative effector proteins that may be associated with *B. bassiana* host flexibility and pathogenesis (Xiao, *et al*., 2012).

Based on our findings, we propose that transgenic *B. bassiana* strains designed to incapacitate the mosquito melanotic response once in the hemolymph may prove to be more potent biocontrol agents than wild-type strains. Finally, the observed delay between PPO localization to hyphal surfaces and melanogenesis renders *B. bassiana* a tractable model to study the yet poorly understood molecular interactions that culminate

in PPO tethering and activation on microbial surfaces; these studies would be difficult to perform in other infection models where the microbe becomes quickly melanized upon contact with the hemolymph, as in the case of *Plasmodium* ookinetes.

C. A serine protease homolog negatively regulates TEP1 consumption in systemic infections of the malaria vector *Anopheles gambiae*

1. Abstract

Clip domain serine protease homologs are widely distributed in insect genomes and play important roles in regulating insect immune responses, yet their exact functions remain poorly understood. Here, we show that CLIPA2, a clip domain serine protease homolog of *Anopheles gambiae*, regulates the consumption of the mosquito complement-like protein TEP1 during systemic bacterial infections. We provide evidence that CLIPA2 localizes to microbial surfaces in a TEP1-dependent manner whereby it negatively regulates the activity of a putative TEP1 convertase, which converts the full-length TEP1-F form into active $TEP1_{cut}$. CLIPA2 silencing triggers an exacerbated TEP1-mediated response that significantly enhances mosquito resistance to infections with a broad class of microorganisms including *Plasmodium berghei*, *Escherichia coli* and the entomopathogenic fungus *Beauveria bassiana*. We also provide further evidence for the existence of a functional link between TEP1 and activation of hemolymph prophenoloxidase during systemic infections. Interestingly, the enhanced TEP1-mediated immune response in *CLIPA2* knockdown mosquitoes correlated with a significant reduction in fecundity, corroborating the existence of a trade-off between immunity and reproduction. In sum, CLIPA2 is an integral regulatory component of the mosquito complement-like pathway which functions to prevent an overwhelming response by the host in response to systemic infections.

2. *Introduction*

Clip domain serine proteases (CLIPs) are central components of extracellular enzymatic cascades in arthropods that regulate several immune responses including antimicrobial peptide expression through the Toll pathway (Lemaitre and Hoffmann, 2007), coagulation (Kurata, *et al*., 2006) and melanization (Cerenius, *et al*., 2008). CLIPs contain a C-terminal chemotrypsin-like serine proteinase domain and 1 or more N-terminal clip domains unique to arthropods. Some CLIPs, also known as serine protease homologs (SPHs), have substitutions of 1 or more residues of their catalytic triad rendering them noncatalytic (Lee, *et al*., 1998). Despite lacking enzymatic activity, SPHs seem to play important regulatory roles in mosquito immune responses. In melanization, efficient cleavage and activation of prophenoloxidase (PPO) by PPO-activating enzymes require SPHs as cofactors (Kwon, *et al*., 2000; Lee, *et al*., 2002; Yu, *et al*., 2003). SPHs have also been involved in cell adhesion (Murugasu, *et al*., 1995) as well as in bacterial opsonization (Lee and Soderhall, 2001). Insect genomes contain several SPHs with clip domains (clip-SPHs): 14 genes were identified in *Drosophila*, whereas 21, 11 and 21 genes were identified in the mosquito vectors *Anopheles gambiae, Aedes aegypti* and *Culex quinquefasciatus*, respectively (Bartholomay, *et al*., 2010; Waterhouse, *et al*., 2007). These genes have undergone extensive diversification between insects as evidenced by the identification of only 3 genes common to the 3 aforementioned mosquito species and 2 genes common to both mosquitoes and *Drosophila* (Bartholomay, *et al*., 2010). Despite the widespread occurrence of clip-SPHs in insect genomes and their implication in a variety of immune responses in different model insects, their modes of action remain poorly understood. Phylogenetic analysis of mosquito clip-SPH sequences revealed 2 subfamilies termed A and E (for CLIPA and CLIPE) with the former containing the majority of genes (Waterhouse, *et*

al., 2007). In the malaria vector *A. gambiae*, SPCLIP1, a member of the CLIPE subfamily, was recently shown to act as a key regulator of the accumulation of the complement C3-like protein TEP1 on surfaces of *Plasmodium* ookinetes and bacteria leading to microbial lysis or melanization (Povelones, *et al.*, 2013). TEP1 is the hallmark of mosquito immune effector responses to both rodent (Blandin, *et al.*, 2004) and human (Dong, *et al.*, 2006; Molina-Cruz, *et al.*, 2013) malaria parasites, as well as to systemic infections with bacteria (Dong, *et al.*, 2006; Levashina, *et al.*, 2001; Moita, *et al.*, 2005) and entomopathogenic fungi (Yassine, *et al.*, 2012). TEP1 exists in the hemolymph as a full-length form called TEP1-F and a proteolytically processed form called $TEP1_{cut}$ that forms a complex with 2 leucine-rich repeat proteins, LRIM1 and APL1C (Fraiture, *et al.*, 2009; Povelones, *et al.*, 2009). SPCLIP1 seems to function as a positive regulator of a yet unidentified TEP1 convertase that catalyzes TEP1-F cleavage to the active $TEP1_{cut}$ form (Povelones, *et al.*, 2013). Here, we show that another clip-SPH of subfamily A, termed CLIPA2, acts as a negative regulator of the mosquito complement-like pathway to control the extent of TEP1-F conversion to $TEP1_{cut}$ during immune responses. We have previously reported that *CLIPA2* knockdown (kd) induces the melanization of *Plasmodium berghei* ookinetes in the basal labyrinth of the midgut epithelium (Volz, *et al.*, 2006). We provide evidence that this phenotype is due to an enhanced TEP1 activity in *CLIPA2* kd mosquitoes which renders them more resistant to bacterial and fungal infections as well. Our data highlight yet an additional level of complexity in the regulation of the mosquito complement-like pathway and inform a broader role of clip-SPHs in insect immune responses.

3. ***Results***

a. <u>*CLIPA2 kd* mosquitoes are more tolerant and resistant to systemic microbial infections</u>

It was previously proposed that CLIPA2 acts as a negative regulator of *A. gambiae* melanization response following the observation that CLIPA2 silencing triggered partial melanization of *P. berghei* ookinetes in the basal labyrinth of the mosquito midgut epithelium (Volz, *et al*., 2006). To gain further insight into CLIPA2 function and its relation to melanization, we monitored the effect of silencing this gene on mosquito susceptibility to systemic bacterial infections with *E. coli* strain OP-50. Using this same strain as a model pathogen, it was suggested that melanization is not required for *A. gambiae* defense against systemic bacterial infections (Schnitger, *et al*., 2007). Hence, if CLIPA2 functions strictly as a negative regulator of melanization, as previously proposed (Volz, *et al*., 2006), then *CLIPA2* kd mosquitoes are expected to show a similar susceptibility to *E. coli* as *Lac*Z kd controls. To address this point, *LacZ, CLIPA2* and *TEP1* kd mosquitoes were injected with live *E. coli* (OD 600 = 0.4), and their survival was scored over a 9-day period. *TEP1* kd mosquitoes served as positive controls since they are susceptible to *E. coli* injections (Dong, *et al*., 2006; Molina-Cruz, *et al*., 2013; Levashina, *et al*., 2001; Moita, *et al*., 2005). Interestingly, *CLIPA2* kd mosquitoes were consistently more tolerant to *E. coli* compared to controls, whereas *TEP1* kd mosquitoes succumbed quickly to the infection as expected (Figure 24A, B). We then measured the resistance of these mosquito genotypes to the injected *E. coli* strain by scoring the bacterial CFUs in mosquito lysates 2 days after injection. Our results revealed that *CLIPA2* kd mosquitoes harbored significantly fewer CFUs than *LacZ* kd controls; mean values were 21,000 and 74,000 CFUs per mosquito, respectively (Figure 24C). In contrast, *TEP1* kd resulted in strong bacterial proliferation with a mean value of 694,000 CFUs per mosquito. Hence, according to our previous

argument, these results do not support a strict role for CLIPA2 in the regulation of melanization because the susceptibility profile of *CLIPA2* kd mosquitoes to *E.coli* deviated significantly from that of *LacZ* kd controls, whereas melanization was previously shown not to affect mosquito tolerance or resistance to the same *E. coli* strain (Schnitger, *et al*., 2007). *CLIPA2* kd mosquitoes also exhibited enhanced tolerance to natural infections with the entomopathogenic fungus *B. bassiana* compared to controls (online suppl. fig. 1; see www.karger.com/doi/10.1159/000363296 for all online suppl. material). In sum, our results suggest that CLIPA2 is likely acting as a negative regulator of a key immune effector response with broad antimicrobial activity, other than melanization.

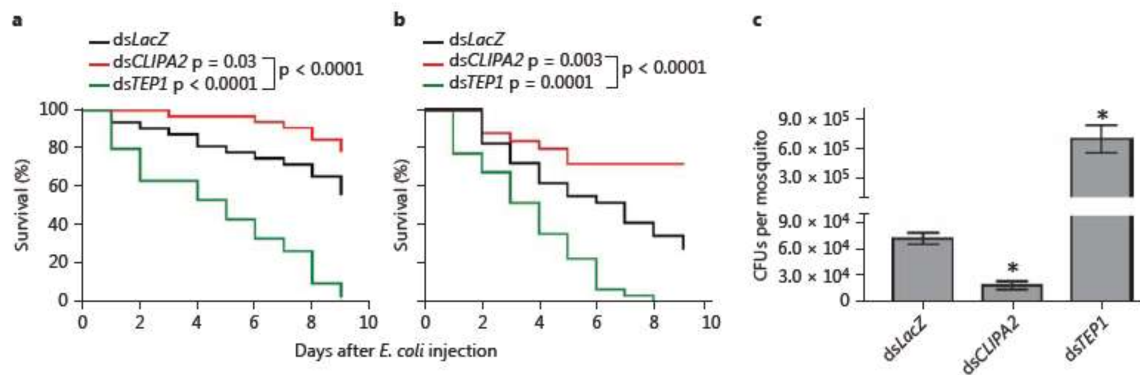


Figure 24. *CLIPA2* kd mosquitoes are more tolerant and resistant to *E. coli* injections. *A. gambiae* adult female mosquitoes were injected with *E. coli* strain OP-50 (OD 600 = 0.4) 4 days after silencing with ds *LacZ* (control), ds *TEP1* and ds *CLIPA2* .(A), (B) Dead mosquitoes were counted daily over a period of 9 days after bacterial challenge. Percent survival was calculated using the Kaplan-Meier method for 2 independent representative experiments. Statistical significance was calculated using the log-rank test. Survival curves were considered to be significantly different if $p < 0.05$. (C) Two days after injection of *E. coli* (OD 600 = 0.4), 5 batches of 8 mosquitoes each per indicated genotype were ground in PBS, and live bacterial counts were determined by plating serial dilutions on LB plates supplemented with ampicillin. Graphs represent mean CFU values per mosquito with their standard errors. Statistical significance was calculated using the Mann-Whitney test, and asterisks indicate $p < 0.05$. Data shown are from 2 independent biological experiments.

b. <u>CLIPA2 controls TEP1 consumption during systemic infections</u>

Based on the phenotype exhibited by *CLIPA2* kd mosquitoes, we hypothesized that CLIPA2 may act as a negative regulator of the complement-like pathway during immune responses for the following reasons: first, TEP1 is, so far, the only effector protein shown to be required for defense against a broad class of microorganisms including *Plasmodium* parasites (Blandin, *et al*., 2004; Dong, *et al*., 2006; Molina-Cruz, *et al*., 2013), bacteria (Dong, *et al*., 2006; Levashina, *et al*., 2001; Moita, *et al*., 2005) and fungi (Yassine, *et al*., 2012); second, *TEP1* kd almost abolished hemolymph PPO activation in response to systemic infections with bacteria (Povelones, *et al*., 2013) and *B. bassiana* (Yassine, *et al*., 2012), suggesting a central role for TEP1 in triggering melanization; third, SPCLIP1, a clip-SPH similar to CLIPA2, acts as a key regulator of the complement-like pathway leading to TEP1 accumulation on microbial surfaces (Povelones, *et al*., 2013). To assess the role of CLIPA2 in regulating mosquito complement we monitored temporally and quantitatively TEP1 protein levels in the hemolymph following mosquito injections with E. *coli* bioparticles. Hemolymph was extracted from *LacZ* and *CLIPA2* kd mosquitoes at 1, 3 and 12 h after injection of *E. coli* bioparticles and subjected to Western blot analysis using TEP1-specific antibodies. In *LacZ* kd mosquitoes, TEP1-F was almost depleted from the hemolymph 1 h after injection but returned to basal levels at later time points (Figure 25A), whereas after silencing *CLIPA2*, TEP1-F levels were markedly reduced up till 3 h after injection and remained below the basal level even at the 12-hour time point. These results indicate that CLIPA2 silencing triggers a more robust and prolonged consumption of TEP1-F in response to systemic bacterial infections. This observed reduction in TEP1-F hemolymph protein is not due to a reduction in *TEP1* gene transcript levels because *CLIPA2* kd mosquitoes exhibited a similar increase in TEP1 transcripts following

bacterial challenge as *LacZ* kd controls (data not shown). In a recent study, SPCLIP1 was shown to positively regulate a complement convertase-like activity (termed TEP1 convertase) responsible for the proteolytic conversion of TEP1-F to $TEP1_{cut}$ (Povelones, *et al.*, 2013). It was clearly demonstrated that *SPCLIP1* silencing abolished infection-induced TEP1-F depletion from the hemolymph (Povelones, *et al.*, 2013). TEP1-F levels in bioparticle-injected *CLIPA2* kd mosquitoes followed completely opposite dynamics with respect to those reported in *SPCLIP1* kd mosquitoes (Povelones, *et al.*, 2013), suggesting a role for CLIPA2 in the negative regulation of TEP1 convertase activity. In that case, *CLIPA2* kd would be expected to lead to increased $TEP1_{cut}$ levels in parallel with the reduction in those of TEP1-F over the examined time range. However, we did not observe such a pattern, but rather $TEP1_{cut}$ was reduced concomitantly with TEP1-F (Figure 25A). This profile was not surprising to us for the following reasons: first, $TEP1_{cut}$ is the active form of TEP1 (Fraiture, *et al.*, 2009; Povelones, *et al.*, 2009); second, $TEP1_{cut}$ localization to microbial surfaces drives several effector responses including opsonization (Levashina, *et al.*, 2001; Moita, *et al.*, 2005), lysis (Blandin, *et al.*, 2004) and melanization (Povelones, *et al.*, 2013; Yassine, *et al.*, 2012). Based on these facts, it is unlikely to detect an increase in levels of $TEP1_{cut}$ in bioparticle-challenged *CLIPA2* kd mosquitoes because it is expected to be consumed in reactions leading to opsonization and melanization of bioparticles. Interestingly, when the same membrane was reprobed with CLIPA2 antibody, CLIPA2 protein was almost depleted from the hemolymph of *LacZ* kd control mosquitoes 1 h after bacterial injection but was replenished at later time points (Figure 25A), indicating a strong consumption of CLIPA2 during immune reactions concomitant with that of TEP1. As expected, CLIPA2 was depleted from the hemolymph of *CLIPA2* kd mosquitoes at all time points after injection showing that CLIPA2 antibody is specific. CLIPA2 migrates

at approximately 100 kDa which is higher than its predicted 66.75 kDa molecular weight (Figure 25A). It should be noted that all protein samples were analyzed under non-reducing conditions, because, for an unknown reason, the antibody failed to recognize CLIPA2 when samples were treated with reducing agents. Since CLIPs have several disulfide bonds, the absence of a reducing agent might have caused this aberrant migration. Alternatively, the presence of a long stretch of threonine residues in CLIPA2 that constitute putative glycosylation sites (online suppl. Figure. 2) may explain this migration pattern. A region of 97 amino acids containing a mucin-like stretch of threonines is absent from the predicted CLIPA2 protein sequence of the PEST strain in VectorBase (www.vectorbase.org) possibly because of an intron misprediction. To our knowledge, this is the first time a mucin-like domain has been identified in a clip domain serine protease. Such domains are known to constitute privileged sites for O glycosylations (Tabak, 2010). Proteins containing mucin-like domains contribute to the formation of extracellular structures including the extracellular matrix (Daley, *et al.*, 2008) and the mucous layer (Dharmani, *et al.*, 2009). The relevance of this threonine-rich region on CLIPA2 structure and function warrants further investigations.

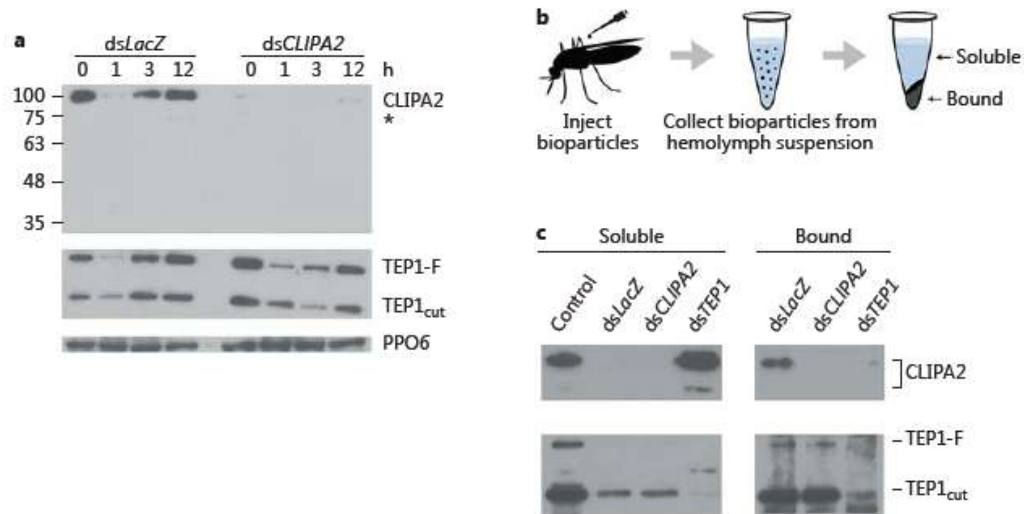


Figure 25. CLIPA2 negatively regulates TEP1 consumption in immune reactions. (A) Western blot analysis of hemolymph extracted from ds *LacZ* and ds *CLIPA2* mosquitoes at the indicated time points after injection of *E. coli* bioparticles. TEP1-F and $TEP1_{cut}$ correspond to the full-length and processed forms of TEP1, respectively. PPO6 was used as a loading control. The asterisk represents a minor CLIPA2 band. (B) Schematic overview of the bioparticle surface extraction assay. Hemolymph containing *E. coli* bioparticles was extracted from the indicated mosquito genotypes 20 min after bioparticle inoculation into the hemocele. Bioparticles were pelleted by centrifugation and the soluble fractions collected. Bacterial pellets were washed with buffer before bound proteins were extracted. (C) Western blot analysis of the soluble and bound fractions using antibodies against CLIPA2 and TEP1. Hemolymph extracted from the same number of un-injected mosquitoes (control lane) served as a reference for comparing the soluble fractions. The image is representative of 3 independent experiments.

c. <u>CLIPA2 is recruited to bacterial surfaces in a TEP1-dependent manner</u>

During infection, TEP1 is recruited to microbial surfaces where it seems to drive several effector responses that culminate in the elimination of the microbe (Povelones, *et al.,* 2013; Blandin, *et al.,* 2004; Levashina; *et al.,* 2001). The concomitant reduction in CLIPA2 and TEP1 protein levels from the hemolymph of *LacZ* kd mosquitoes 1 h after bioparticle injection (Figure 25A) suggests that CLIPA2 is likely to be sequestered on bacterial surfaces together with TEP1. We addressed this point using the bioparticle surface extraction assay that allows the quantitative assessment of interactions occurring between mosquito hemolymph proteins and bacterial surfaces, as

previously described (Povelones, *et al.,* 2013). Briefly, *E. coli* bioparticles were injected into female mosquitoes, and hemolymph was extracted 15 min after injection. Bioparticles were separated from the hemolymph by centrifugation, washed extensively and their surface-bound proteins eluted for Western blot analysis (Figure 25B and C). In *LacZ* kd controls, CLIPA2 was almost exclusively detected in the bound fraction with a barely detectable band appearing in the soluble one (Figure 25C). Interestingly, in *TEP1* kd mosquitoes CLIPA2 distribution between both fractions was completely reversed; CLIPA2 was almost absent from the bound fraction and enriched in the soluble fraction, indicating that TEP1 is required for CLIPA2 recruitment to bioparticle surfaces. In *LacZ* kd controls, both $TEP1_{cut}$ and TEP1-F were more enriched in the bound fraction, with $TEP1_{cut}$ being by far more abundant (Figure 25C). These results conform with previous observations showing that $TEP1_{cut}$ is the main form associated with bacterial surfaces (Povelones, *et al.,* 2013; Levashina, *et al.,* 2001). In *CLIPA2* kd mosquitoes, $TEP1_{cut}$ and TEP1-F levels were similar to those in *LacZ* kd controls in both the soluble and bound fractions clearly indicating that TEP1 acts upstream of CLIPA2. Based on the data in figure 25A, TEP1 cut was expected to be more abundant in the bound fraction of *CLIPA2* kd mosquitoes relative to *LacZ* kd controls, but this was not the case. A plausible explanation is that excess $TEP1_{cut}$ accumulating on bioparticle surfaces in the former genotype triggered their rapid elimination through opsonisation and melanization. These immune reactions render bioparticles nonextractable due to their uptake by hemocytes in the abdomen (King and Hillyer, 2012) or adherence to tissues. In support of this argument, we consistently obtained smaller bioparticle pellets after the centrifugation of hemolymph extracts from *CLIPA2* kd mosquitoes relative to those from the same number of *LacZ* kd controls (data not shown), indicating a quicker elimination of microbes in the former genotype.

d. *CLIPA2 kd* enhances hemolymph phenoloxidase activity in response to systemic bacterial infections

Bacterial injections into the hemocele of wild-type *A. gambiae* mosquitoes triggered PPO activation leading to melanization (Schnitger, *et al.,* 2009; 2007). TEP1 controls tightly the activation of hemolymph PPO, since TEP1 silencing almost abolished PPO activation following bacterial (Povelones, *et al.,* 2013) and fungal (Yassine, *et al.,* 2012) systemic infections. Furthermore, TEP1 depletion inhibited the infection-induced cleavage of CLIPA8, an essential factor for PPO activation (Povelones, *et al.,* 2013). Consequently, if CLIPA2 is acting indeed as a negative regulator of TEP1 consumption, then silencing its gene is expected to trigger an exacerbated melanotic response to systemic bacterial infections. To address this point, we first scored the intensity of melanization in abdomens of *LacZ* and *CLIPA2* kd mosquitoes dissected 48 h after injection of live *E. coli,* and grouped abdomens into 4 empirical categories depending on the size and spatial distribution of melanotic foci. In *LacZ* kd mosquitoes, the majority of dissected abdomens showed small melanotic foci restricted to the periosteal regions (Figure 26A, C). Periosteal hemocytes were shown to aggregate in these regions during systemic infections in order to sequester microbes present in the hemolymph (King and Hillyer, 2012). However, the majority of abdomens from *CLIPA2* kd mosquitoes showed larger melanotic foci with a more scattered distribution (Figure 26B, C) indicating an exacerbated melanotic response. To further support these observations, phenoloxidase activity was measured in *LacZ* , *CLIPA2* and *TEP1* kd mosquitoes 3 h after injection of live *E. coli* ($O.D._{600} = 0.8$). *CLIPA2* kd mosquitoes exhibited a significant 2.4-fold increase in phenoloxidase activity compared to *LacZ* controls (Figure 26D), which explains the intense bacterial melanization observed in their abdomens. *TEP1* kd almost abolished PPO activation as previously reported (Povelones, *et al.,* 2013). In sum, these results strongly suggest that

enhanced TEP1 consumption in systemic infections of *CLIPA2* kd mosquitoes is responsible for the abnormal phenoloxidase activity measured in their hemolymph.

e. <u>CLIPA2 is a component of the mosquito complement-like pathway</u>

$TEP1_{cut}$ is stabilized in the hemolymph by a complex of 2 leucine-rich repeat proteins LRIM1 and APL1C (Fraiture, *et al.,* 2009; Povelones, *et al.,*2009). Silencing either of these proteins in naive mosquitoes triggered the loss of $TEP1_{cut}$ from the hemolymph compartment and its accumulation on host tissues. Interestingly, TEP1 cut accumulation on tissues was concomitant with almost the depletion of SPCLIP1 from the hemolymph, indicating that SPCLIP1 is tightly linked to the $TEP1_{cut}$ function (Povelones, *et al.,* 2013). Thus, we examined whether CLIPA2 steady-state levels in the hemolymph show similar dependency on $TEP1_{cut}$ under naïve conditions. Naive mosquitoes were injected with dsRNAs corresponding to the indicated genes, and hemolymph was extracted 48 h later and subjected to Western blot analysis. *TEP1* kd showed no effect on CLIPA2 steady-state levels which were similar to those in the control group (Figure 27). However, *LRIM1* kd almost abolished CLIPA2 presence in the hemolymph concomitant with the loss of $TEP1_{cut}$ (Figure 27), suggesting that CLIPA2 is following the latter to the tissues. Cosilencing TEP1 and LRIM1 restored the baseline levels of CLIPA2 suggesting that it functions downstream of $TEP1_{cut}$. *CLIPA2* kd did not affect the steady-state levels of LRIM1 or TEP1. Altogether, these results clearly indicate that, similar to SPCLIP1 (Povelones, *et al.,* 2013), CLIPA2 is tightly linked to the $TEP1_{cut}$ function in both infected and naïve conditions, and it constitutes as such an integral component of the TEP1 pathway.

f. *CLIPA2* silencing exacerbates TEP1-mediated killing of *P. berghei* ookinetes

CLIPA2 silencing was previously shown to trigger partial melanization of *P. berghei* ookinetes in susceptible mosquitoes of strain G3 (Volz, *et al.,* 2006). Based on the data presented so far, a plausible explanation to this phenotype is an exaggerated TEP1-mediated killing of ookinetes leading to melanization. Before addressing this point, we first reassessed the effect of CLIPA2 silencing on ookinete melanization in our G3 mosquitoes for the following reasons: first, the melanotic phenotype previously reported for *CLIPA2* silencing was partial (8% melanized ookinetes) (Volz, *et al.,* 2006); second, the colony has probably passed through several bottlenecks which might have altered the *CLIPA2* kd phenotype. Therefore, *LacZ* , *CLIPA2* and *TEP1* kd mosquitoes were allowed to feed on *P. berghei*-infected mice and their midguts dissected 7 days later to score for melanized ookinetes and live oocysts. *CLIPA2* kd mosquitoes exhibited a significant increase in numbers of melanized ookinetes (p Mo<0.001) concomitantly with a reduction in numbers of live oocysts (p Lo <0.001; (Figure 28A, B) relative to controls. However, the numbers of melanized ookinetes were significantly higher in our current G3 mosquitoes than those reported previously in the same strain (56.2 vs. 8%, respectively), possibly due to genotypic changes at specific loci resulting from colony bottlenecks. *TEP1* kd, used as a positive control, triggered a significant increase in numbers of live oocysts as previously reported (Blandin, *et al.*, 2004).To determine whether CLIPA2 regulates the extent of ookinete killing by TEP1, we examined the effect of silencing *CLIPA2* on the numbers of TEP1-stained ookinetes. Accordingly, midguts were dissected from *LacZ* and *CLIPA2* kd mosquitoes at 26 h after an infectious blood meal and immunostained with TEP1 antibody. The numbers of ookinetes positive for GFP (GFP+), TEP1 (TEP+), both GFP and TEP1 (GFP+/TEP+) or melanin (i.e. melanized) were scored using confocal

microscopy (Figure 28 C, D). Midguts dissected from *CLIPA2* kd mosquitoes showed an overall significant increase in TEP1+ parasites (TEP1+ and GFP+/TEP1+ and melanized) in comparison to controls, in 2 independent biological experiments (Figure 28E): 63.9 versus 46.1% in experiment 1 and 59.8 versus 25.6% in experiment 2, respectively. Melanized ookinetes were considered TEP1+ since TEP1 is required for ookinete melanization, and PPO localizes only on the surface of TEP1-labeled ookinetes (Blandin, *et al.,* 2004). Hence, these results suggest that the enhanced ookinete killing in *CLIPA2* kd mosquitoes, manifested in the form of melanized ookinetes, is due to an exaggerated TEP1-mediated response to these parasite stages.

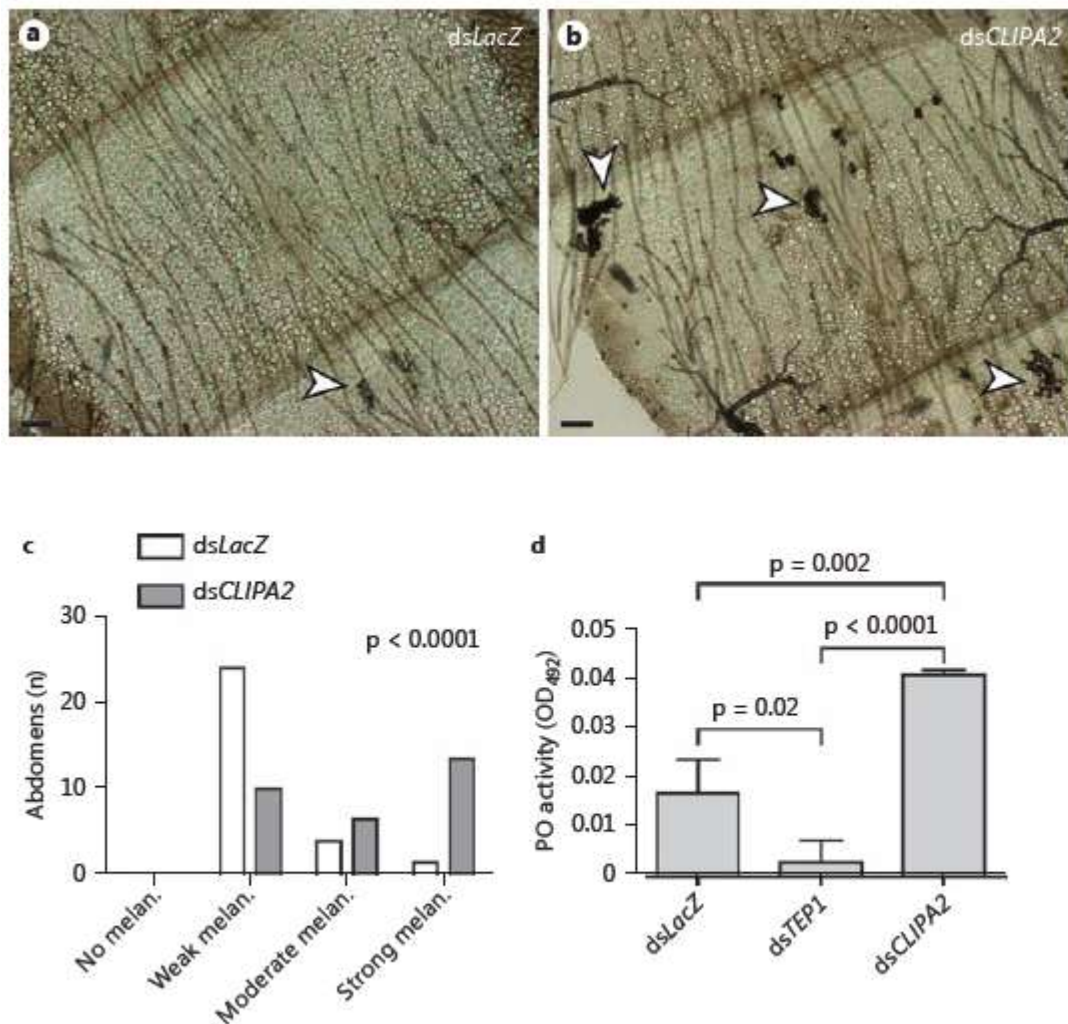


Figure 26. *CLIPA2* kd enhances the mosquito melanotic response to injected *E. coli.* Abdomens were dissected from ds *LacZ* and ds *CLIPA2* mosquitoes 48 h after

injection of *E. coli* strain OP-50 (O.D. $_{600}$ = 0.4), and the intensity of melanization (melan.) was scored empirically as described in the Methods section. Bright-field images of ds *LacZ* (A) and ds *CLIPA2* (B) abdomens show weak and strong bacterial melanization phenotypes, respectively. Arrowheads indicate melanized clusters of bacteria. Scale bars are 10 µm. (C) Distribution of melanization intensity scored in dissected abdomens pooled from 2 independent biological experiments. Distributions were compared using the Kolmogorov-Smirnov test. (D) Phenoloxidase (PO) enzymatic activity (detected as absorbance at 492 nm, O.D. 492 , after conversion of L -3,4-dihydroxyphenylalanine) was measured in hemolymph extracted from ds *CLIPA2* and ds *TEP1* mosquitoes and compared to that in ds *LacZ* controls 3 h after injection of *E.coli* (O.D.$_{600}$ 0.8). The graphs show phenoloxidase activity measured 40 min after addition of L -3,4 dihydroxyphenylalanine. Means were calculated from 3 independent biological experiments. Error bars represent standard deviation of the mean. Statistical analysis was performed using Student's t test, and differences were considered to be significant if $p < 0.05$.

g. *CLIPA2 kd* reduces mosquito fecundity in response to systemic bacterial infections

An exacerbated immune response may affect host fitness through the redistribution of energy resources allocated to other vital physiological processes or through reduced tolerance to the microbe driven by immune-mediated tissue damage (Ayres and Schneider, 2012). *CLIPA2* kd mosquitoes trigger an enhanced TEP1-mediated immune response to bacterial, fungal and *Plasmodium* infections yet they seem to tolerate or endure the infection even better than controls, at least in terms of their survival rate. Since survival is one among several fitness related parameters, we looked for other hidden costs in particular reproductive success especially that previous studies in *A. gambiae* pointed to the existence of a tradeoff between ookinete killing and oogenesis (Rono, *et al.,* 2010). To address this point, *LacZ* and *CLIPA2* kd mosquitoes were injected with an *E.coli* suspension (O.D $_{600}$ 0.4) and allowed to recover during 4 days after the injection process. Wild-type mosquitoes injected with sterile PBS were also given the same recovery period. Non-injected naive mosquitoes were used as controls. Four days after recovery, mosquitoes from the 4 experimental groups were fed on the same naive mouse for an equal amount of time, and eggs laid by individually

sorted females were counted using a stereoscope. Injection of sterile PBS reduced significantly mosquito fecundity relative to control, suggesting that wounding per se reduces fecundity (figure 29). *LacZ* kd mosquitoes injected with *E.coli* exhibited further reduction in egg production compared to the PBS-injected group ($p < 0.0001$), possibly due to additional cost imposed by infection and immune activation. Interestingly, *E.coli*-injected *CLIPA2* kd mosquitoes produced even fewer eggs than their *LacZ* kd controls ($p < 0.0001$). Since *CLIPA2* silencing renders mosquitoes more resistant to systemic *E.coli* injections (figure 24C), the lower fecundity of these genotypes reflects most probably the cost of triggering an overwhelming TEP1-mediated systemic response.

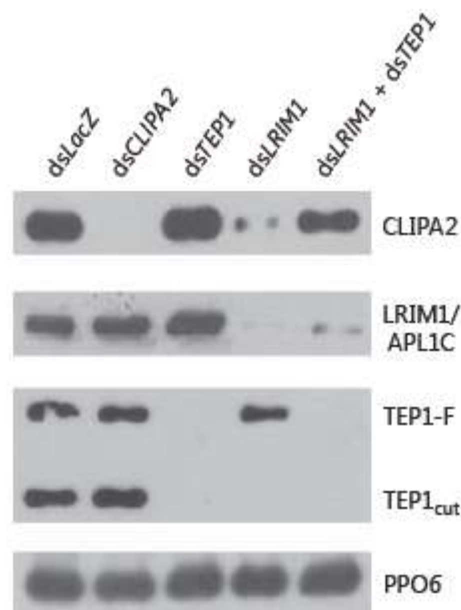
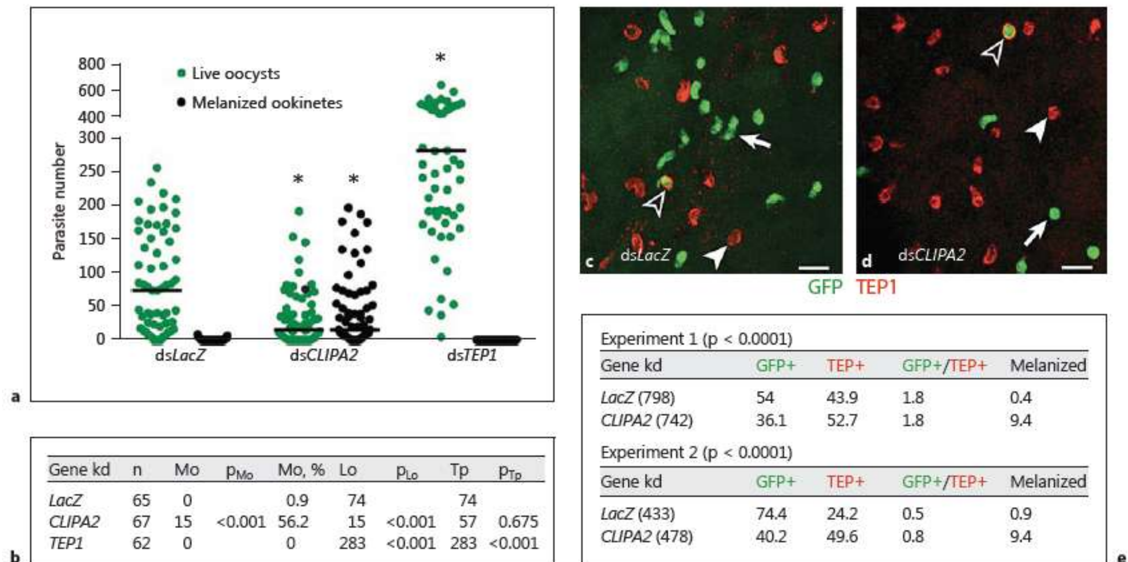


Figure 27. CLIPA2 is a component of the complement-like pathway. Western blot analysis of hemolymph extracted 2 days after injecting naive mosquitoes with the indicated dsRNAs. The membrane was probed with antibodies against CLIPA2, LRIM1, TEP1 and PPO6 as loading control. The image is representative of 3 independent biological experiments.



Gene kd	n	Mo	p_{Mo}	Mo, %	Lo	p_{Lo}	Tp	p_{Tp}
LacZ	65	0		0.9	74		74	
CLIPA2	67	15	<0.001	56.2	15	<0.001	57	0.675
TEP1	62	0		0	283	<0.001	283	<0.001

Experiment 1 (p < 0.0001)

Gene kd	GFP+	TEP+	GFP+/TEP+	Melanized
LacZ (798)	54	43.9	1.8	0.4
CLIPA2 (742)	36.1	52.7	1.8	9.4

Experiment 2 (p < 0.0001)

Gene kd	GFP+	TEP+	GFP+/TEP+	Melanized
LacZ (433)	74.4	24.2	0.5	0.9
CLIPA2 (478)	40.2	49.6	0.8	9.4

Figure 28. TEP1-mediated ookinete killing is increased in *CLIPA2* kd mosquitoes. (A) Scatter plot of live GFP-expressing *P. berghei* oocysts (green circles) and dead melanized ookinetes (black circles) scored in the midguts of ds *LacZ* , ds *CLIPA2* and ds *TEP1* mosquitoes 7 days after infection. Lines indicate median parasite numbers. The Mann-Whitney test was used to compare ds *CLIPA2* and ds *TEP1* mosquitoes with ds *LacZ* controls, and asterisks indicate p-values less than 0.05. Data were pooled from 3 independent biological experiments. (B) Tabulated data of (A) showing the median numbers of melanized ookinetes (Mo), live oocysts (Lo) and total parasites (Tp) per midgut. P-values were calculated by the Mann-Whitney test. (C), (D) Representative confocal projections from ds *LacZ* (C) and ds *CLIPA2* (D) mosquito midguts dissected 26 h after infection with *P. berghei* . Live *P. berghei* ookinetes expressing GFP are green (arrows). TEP1-stained (red) ookinetes are fragmented and have lost the GFP signal (filled arrowheads). There is an increased number of TEP1-stained ookinetes (dead) in midguts of ds *CLIPA2* mosquitoes relative to ds *LacZ* controls. Note the rare presence of ookinetes that are TEP1+ and GFP+ (open arrowheads), and which are most likely in the early death phase. Scale bars are 10 µm. (E) Ookinete percentages in the 4 indicated groups [GFP+ (live), TEP1+ (dead), GFP+/TEP1+ (dying) and melanized (dead)] from 2 independent biological experiments. Approximately, 10 midguts were processed per genotype in each experiment. The total number of ookinetes scored per genotype is shown in parentheses. p values were calculated by the χ^2 analysis comparing percentages of TEP1+ ookinetes (these include TEP+, GFP+/TEP+ and melanized) between the 2 mosquito genotypes.

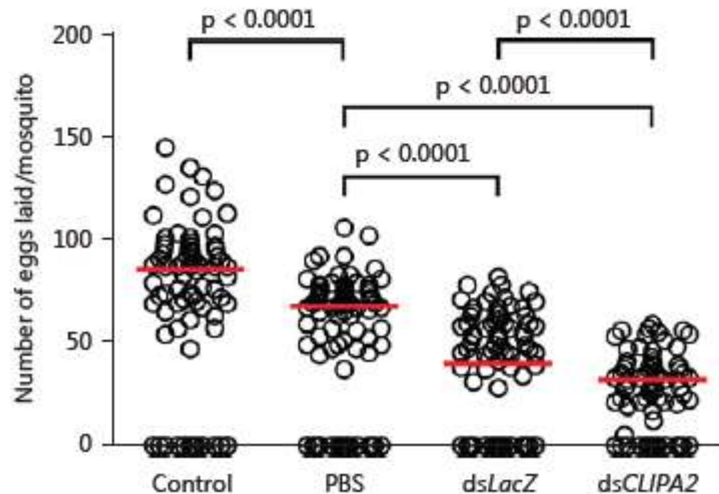


Figure 29. Infection-induced reduction in fecundity is further enhanced in *CLIPA2* kd mosquitoes. Mosquitoes treated with ds *LacZ* and ds *CLIPA2* were injected with live *E. coli* (OD 600 = 0.4). Noninjected and PBS-injected wild-type naive mosquitoes served as controls. All experimental groups were fed on the same naïve mouse for an equal period of time. The scatter plot represents the median number (red lines) of eggs per mosquito in each experimental group. Statistical analysis was performed using the Mann-Whitney test. Medians were considered significant if $p < 0.05$.

4. Discussion

Clip-SPHs play important regulatory roles in insect immune responses. Here, using the bacterial bioparticle infection system we demonstrate that CLIPA2 localizes to microbial surfaces whereby it negatively regulates the consumption of mosquito TEP1 during systemic infections, adding a new level of complexity to complement regulation in *A. gambiae.* CLIPA2 functions to avoid an overwhelming TEP1-mediated response that may be energetically costly for the host. Such a response is indeed taking place in *CLIPA2* kd mosquitoes according to the following observations: abnormally high hemolymph phenoloxidase activity in response to systemic *E. coli* injections; enhanced tolerance to *E. coli* and *B. bassiana* infections, and increased resistance to *E. coli* and *P. berghei* ookinetes. Unfortunately, we were not able to investigate the potential

interaction between CLIPA2 and TEP1 in vivo by immunoprecipitation assays, since the CLIPA2 antibody in hand does not recognize the native protein. This also hampered efforts to localize CLIPA2 on ookinete surfaces using fluorescent microscopy. However, based on our data, we expect CLIPA2 to colocalize with TEP1 on surfaces of *Plasmodium* ookinetes as well as on other microbes coming in contact with the hemolymph. This is supported by the fact that CLIPA2 loss from the hemolymph paralleled that of TEP1 in 2 different experimental settings: bioparticle-injected *LacZ* kd mosquitoes (Figure 26A) and *LRIM1* kd naive mosquitoes (Figure 27). The concomitant loss of CLIPA2 and $TEP1_{cut}$ from the hemolymph of *LRIM1* kd mosquitoes and the rescue of CLIPA2 loss after cosilencing LRIM1 and TEP1 indicate that CLIPA2 acts downstream of TEP1, and it is an integral component of the mosquito complement-like pathway. CLIPA2 is the second protein after SPCLIP1 shown to be depleted from the hemolymph of *LRIM1* kd mosquitoes as a consequence of $TEP1_{cut}$ deposition on tissues, suggesting that this loss of $TEP1_{cut}$ is not a mere form of protein precipitation as previously suggested (Fraiture, *et al.,* 2009) but is rather indicative of an autoimmune attack. Unidentified tissue-specific factors likely act to prevent this attack from proceeding to a full-blown response causing immunopathology since TEP1-F levels remain unchanged after silencing LRIM1 (Figure 27) (Povelones, *et al.,* 2013). Additionally, melanization has not been observed in abdomens of *LRIM1* kd mosquitoes [Osta M.A., pers. commun.]. The loss of CLIPA2 from the hemolymph of bioparticle-challenged *dsLacZ* mosquitoes is replenished later probably through the action of immune signaling pathways controlling the activation of NF-κB, STAT (Reviewed in Clayton, *et al.,* 2014) or AP-1 (Garver, *et al.,* 2013) transcription factors, which all have been shown to be involved in mosquito immunity. Which of these pathways contributes most to CLIPA2 replenishment remains to be elucidated. It is intriguing that the two

CLIPs that were shown so far to regulate TEP1-F consumption are noncatalytic, which raises the possibility that they might be regulating the efficiency of TEP1-F cleavage. Le, *et al.,* 2012 showed that limited activation cleavage of recombinant TEP1-F in vitro resulted in a meta $TEP1_{cut}$ form which slowly and spontaneously converted to mature $TEP1_{cut}$ that is captured and stabilized by the LRIM1/APL1C complex. $TEP1_{cut}$ bound to microbial surfaces may originate from two different sources (Figure 30): the first is the LRIM1/APL1C complex that is thought to deliver its $TEP1_{cut}$ cargo onto microbial surfaces (Fraiture, *et al.,* 2009; Povelones, *et al.,* 2009; Baxter, *et al.,* 2010). The second source is a putative TEP1 convertase triggered locally by $TEP1_{cut}$ originating from the LRIM1/APL1C complex (Povelones, *et al.,* 2013). Once formed this convertase is thought to convert TEP1-F into mature $TEP1_{cut}$, hence amplifying the response. In this context, our data suggest a role for CLIPA2 in the negative regulation of TEP1 convertase, thus controlling the extent of TEP1-F conversion to $TEP1_{cut}$ on microbial surfaces. Since CLIPA2 lacks catalytic activity, it is tempting to speculate that it might also act as a molecular chaperon to regulate the conversion of meta $TEP1_{cut}$ to the mature form *in vivo*. CLIPA2 contains 2 N-terminal clip domains suggesting its potential interaction with multiple proteins in the TEP1 pathway. The clip domain has been shown to serve as a module essential for binding cleaved phenoloxidase and the subsequent formation of active phenoloxidase clusters (Huang, *et al.,* 2007; Piao, *et al.,* 2005). The future decoding of the CLIPA2 interactome is expected to reveal additional insight into its exact role in the mosquito complement-like pathway.

When survival (i.e. health) was used as a parameter to measure mosquito tolerance to infection, *CLIPA2* kd mosquitoes exhibited increased tolerance to systemic *E. coli* injections and natural *B. bassiana* infections, compared to controls. Tolerance 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). A plausible explanation for the increased tolerance of *CLIPA2* kd mosquitoes is the quick clearance or control of microbial growth triggered by the enhanced TEP1-mediated response, possibly limiting infection-induced tissue damage. There is increasing evidence that tissue damage can affect host tolerance to infection (Jamieson, *et al.,* 2013). However, when reproductive success was considered as a parameter to measure the tolerance of *CLIPA2* kd mosquitoes to systemic *E. coli* infections, a reduced tolerance was noted, suggesting the existence of a trade-off between TEP1-mediated immunity and fecundity. In fact, the first molecular link between these two processes was provided by Rono, *et al.,* 2010 who showed that depletion of the nutrient transport proteins vitellogenin and lipophorin, required for oogenesis, enhanced TEP1-mediated killing of *P. berghei* ookinetes in midguts of *A. gambiae* mosquitoes (Rono, *et al.,* 2010). It remains to be determined how the enhanced TEP1-mediated response in *CLIPA2* kd mosquitoes reduced fecundity and whether it entails redistribution of energy resources or altering the expression of nutrient transport proteins.

Another interesting result that emerged from our study is the functional link between mosquito complement and PPO activation. We recently showed that hemolymph PPO activation following systemic bacterial injections as well as the activation cleavage of CLIPA8, a key regulator of the mosquito melanization response (Yassine, *et al.,* 2012; Volz, *et al.,* 2006; Schnitger, *et al.,* 2007), require the functions of both, TEP1 and SPCLIP1, suggesting the existence of a functional link between TEP1 and PPO activation (Povelones, *et al.,* 2013). Here, we provide further evidence in support of this link by showing that the enhanced TEP1-mediated response in *CLIPA2* kd mosquitoes was paralleled by an abnormal increase in hemolymph phenoloxidase activity. Based on these observations, we propose that in *A. gambiae* the

infection-induced melanization response may constitute an effector arm of the complement like pathway. It remains to be elucidated how TEP1 triggers PPO activation at the molecular level and why *Plasmodium* ookinetes rarely become melanized in susceptible mosquitoes despite being bound by TEP1, while bacteria (Povelones, *et al.,* 2013; Schnitger, *et al.,* 2007) and fungi (Yassine, *et al.,* 2012) are efficiently melanized.

In conclusion, there is increasing evidence that TEP1 consumption during systemic immune responses is subject to tight regulation possibly to establish a balance between an efficient immune response on one hand and minimal fitness costs on the other. Identifying the components of this regulatory network is essential if we are to build a comprehensive model of the activation and regulation of this ancient complement-like system and define to which extent common strategies exist with that of mammals.

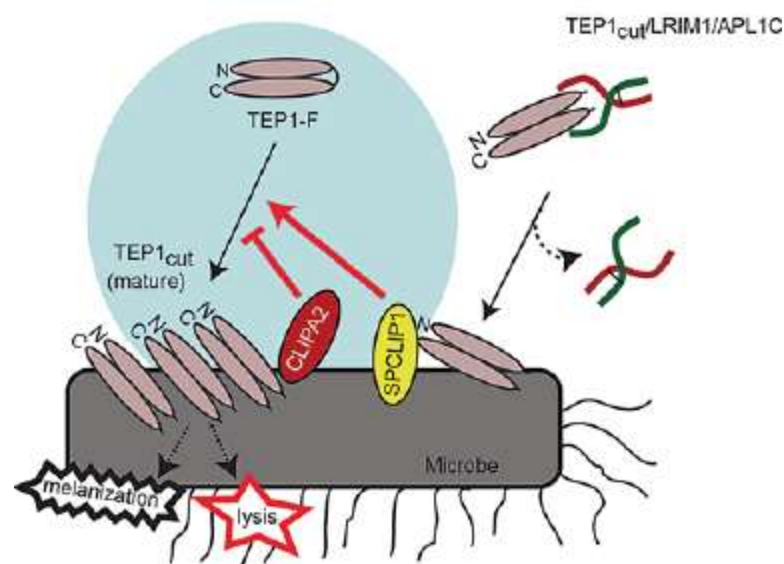


Figure 30. Model of CLIPA2 function. $TEP1_{cut}$ released from the LRIM1/APL1C complex (shown in red and green) on microbial surfaces triggers the formation of a putative convertase which requires SPCLIP1 function to process TEP1-F into more $TEP1_{cut}$ .Mature $TEP1_{cut}$ binds to microbial surfaces initiating several effector reactions including lysis and melanization. CLIPA2 seems to function as a negative regulator of the putative convertase, hence controlling the extent of TEP1-F processing. The N- and C-terminal domains of TEP1 are shown as elliptic structures. The convertase activity is highlighted by a blue circle.

D. Functional interactions between apolipophorins and complement regulate the mosquito immune response to systemic infections

1. Abstract

The complement-like protein TEP1 is the hallmark effector molecule against *Plasmodium* ookinetes in the malaria vector *Anopheles gambiae*. TEP1-mediated ookinete killing requires several steps including stabilization of active TEP1 and directing TEP1 to ookinete surfaces. We have previously revealed yet another level of regulation over TEP1, whereby knockdown (kd) of the non-catalytic clip domain serine protease *CLIPA2* increased TEP1 accumulation on ookinetes surfaces and rendered mosquitoes more resistant to *Plasmodium*, bacterial and fungal infections. Here, CLIPA2 co-immunoprecipitation from the hemolymph of *Beauveria bassiana* infected mosquitoes followed by mass spectrometry and functional genetic analysis, lead to the identification of *Apolipophorin-II/I* gene, encoding the two lipid carrier proteins Apo-I and II, as a novel negative regulator of TEP1-mediated immune response during mosquito systemic infections. *Apo-II/I* exhibits a similar RNAi phenotype as *CLIPA2* in mosquito bioassays characterized by increased resistance to *B. bassiana* and *Escherichia coli* infections. We provide evidence that this enhanced resistance to systemic infections is TEP1-dependent. By monitoring GFP release in the hemolymph from a GFP-expressing strain of *E. coli* we provide direct evidence for the role of TEP1 in bacterial lysis and show that this lytic activity is enhanced in *Apo-II/I* and *CLIPA2* kd mosquitoes in a TEP1-dependent manner. Interestingly, silencing *Apo-II/I* but not *CLIPA2* upregulated the expression of *TEP1* following systemic infections with *E. coli* and *B. bassiana* in a c-Jun N-terminal kinase (JNK) pathway dependent manner. Our results suggest that mosquito Apolipophorins-II/I play an important immune regulatory

role during systemic infections and provide novel insight into the functional interplay between lipid metabolism and immune gene regulation.

2. Introduction

Mosquitoes like other invertebrates lack adaptive immunity and depend solely on the innate immune system to defend themselves against microbial infections. The recognition of pathogen associated molecular patterns (PAMPs) by mosquito pattern recognition receptors (PRRs) triggers the downstream activation of several immune effector mechanisms that clear the infecting agent. In the malaria vector *Anopheles gambiae*, the complement-like attack mediated by the thioester-containing protein 1 (TEP1) was shown to be a hallmark effector response against *Plasmodium* ookinetes invading the midgut epithelium (Blandin, *et al.*, 2004) and against systemic infections with bacteria (Dong, *et al.*, 2006) and fungi (Yassine, *et al.,* 2012). TEP1 is produced by hemocytes and binds to the surface of ookinetes as they emerge from midgut epithelial cells into the basal labyrinth of the gut epithelium triggering their death presumably through lysis. It is also required for the melanization of ookinetes in certain refractory mosquito genotypes (Blandin, *et al.*, 2004; Shiao, *et al.*, 2006). TEP1 also binds to the surfaces of bacteria (Levashina, *et al.*, 2001) and fungal hyphae (Yassine, *et al.*, 2012) triggering their phagocytosis and melanization, respectively. More recently, it was shown that TEP1 is expressed in the male testes where it plays an essential role in the removal of defective apoptotic sperm cells during spermatogenesis, a process crucial for male fertility (Pompon and Levashina, 2015). Due to its key role in mosquito immunity, TEP1 is tightly regulated at the transcriptional and activation levels. The basal levels of TEP1 were initially shown to be controlled by the *Rel1* and *Rel2* signaling pathways (Frolet, *et al.*, 2006). Enhancing TEP1 expression by silencing the negative regulators

of these pathways increased mosquito resistance to *Plasmodium* infections (Frolet, *et al.*, 2006; Garver, *et al.*, 2009). A more recent report revealed a significant role for the JNK pathway in controlling the basal expression levels of TEP1 in hemocytes (Garver, *et al.*, 2013), suggesting that multiple transcription factors act in concert to control TEP1 promoter activity. At the activation level, full-length TEP1 (TEP1-F) is cleaved in the hemolymph by an unknown protease into active $TEP1_{cut}$ which is stabilized by a complex of two leucine-rich immune proteins, APL1C and LRIM1 (Fraiture, *et al.*, 2009; Povelones, *et al.*, 2009). APL1C/LRIM1 complex controls the precocious activation of TEP1 in the hemolymph and is required for its binding to *Plasmodium* ookinetes. It was shown later, that nitration of ookinetes by heme peroxidase 2 (HPX2) and NADPH oxidase 5 (NOX5) during the traversal of midgut epithelial cells directs TEP1 to their surfaces (Oliveira, *et al.*, 2012). HPX2 is also required for TEP1 binding to apoptotic sperm cells in mosquito males (Pompon and Levashina, 2015), suggesting that nitration is probably a prerequisite for TEP1 binding to all its target cells. Following activation, TEP1 accumulation on microbial surfaces is also controlled by two non-catalytic clip domain serine proteases (CLIPs), SPCLIP1 and CLIPA2, which act as positive and negative regulators of that process, respectively, suggesting the presence of a convertase-like activity analogous to that in mammalian complement. Silencing *LRIM1* in naive mosquitoes triggered the loss of $TEP1_{cut}$ (Fraiture, *et al.*, 2009; Povelones, *et al.*, 2009), SPCLIP1 (Povelones, *et al.*, 2013) and CLIPA2 (Yassine, *et al.*, 2014) from the hemolymph, demonstrating the tight association of these CLIPs with TEP1 activity.

CLIPA2 has a particular RNAi phenotype characterized by increased mosquito resistance to infections with *Plasmodium*, bacteria and fungi, in addition to a reduction in egg laying. Here, using CLIPA2 co-immunoprecipitation (coIP) followed by mass

spectrometry and functional genetic analysis, we identified Apolipophorins-I and II as novel regulators of mosquito complement during systemic infections with bacteria and fungi. *Apo-II/I* silencing increased mosquito resistance to bacterial and fungal infections in a TEP1-dependent manner. It was previously reported that *Apo-II/I* kd mosquitoes exhibit increased resistance to *Plasmodium* infections in a manner also dependent on TEP1 (Rono, *et al.*, 2010), suggesting that Apo-I and II play a broad regulatory role in mosquito immunity. We provide evidence that silencing *Apo-II/I* upregulated the expression of *TEP1* following systemic infections with *E. coli* and *B. bassiana* in a c-Jun N-terminal kinase (JNK) pathway dependent manner. Our data highlight a novel functional link between lipid carrier proteins, complement and JNK signaling in regulating mosquito immune responses during systemic infections.

3. Results

a. Apo-II/I proteins novel negative regulators of mosquito immune responses to systemic infections

TEP1 is so far regulated by two classes of proteins: the leucine-rich immune proteins LRIM1 and APL1C form a heterodimeric complex that binds to and stabilizes $TEP1_{cut}$, the active form of TEP1, in the mosquito hemolymph (Fraiture, *et al.*, 2009; Povelones, *et al.*, 2009), while the two non-catalytic CLIPs, SPCLIP1 (Povelones, *et al.*, 2013) and CLIPA2 (Yassine, *et al.*, 2014) regulate positively and negatively $TEP1_{cut}$ accumulation on microbial surfaces, respectively. In order to provide insight into the functional interaction between CLIPA2 and TEP1 and in the aim of identifying novel complement regulators with similar roles to CLIPA2, we set out to determine the identity of proteins that coIP with CLIPA2 from the hemolymph of *B. bassiana* infected mosquitoes using mass spectrometry (MS). The strategy of coupling coIP with MS was

used as a gene discovery approach in order to obtain an unbiased, broad view of the interactions of CLIPA2 with hemolymph proteins. There are two important reasons behind choosing *B. bassiana* as the main model infectious agent in this assay and throughout: first, it follows a natural infection route following mosquito spraying with spores (Yassine, *et al.*, 2012) eliminating the wounding effect generated with bacterial injections; second, *B. bassiana* infections were shown to trigger a potent immune response in the mosquito involving TEP1 (Yassine, *et al.*, 2012). At first, a small scale CLIPA2 coIP was performed from hemolymph collected from 100 mosquitoes at 48 hrs after natural infection with *B. bassiana* [the time when the infection is well established (Yassine, *et al.*, 2012)] to test the efficiency of the affinity purified CLIPA2 antibody cross-linked to magnetic beads in immunoprecipitating CLIPA2, and determine whether we can detect interactions with certain components of the mosquito complement pathway for which antibodies are available. Beads lacking antibody and naive mosquitoes served as controls. The results revealed that αCLIPA2 almost depleted CLIPA2 from the hemolymph of naive mosquitoes while no interactions were detected with control beads highlighting the specificity of the interaction (Figure 31). When the same experiment was repeated with hemolymph collected from the same number of mosquitoes infected with *B. bassiana*, CLIPA2 was not completely immunoprecipitated using the same volume of αCLIPA2 beads, suggesting that infection is most likely increasing CLIPA2 expression. Reprobing the membrane with available antibodies revealed that $TEP1_{cut}$ co-immunoprecipitated with CLIPA2 under infected and non-infected conditions. SPLCIP1 was also detected in both conditions, but not the CTL4/CTLMA2 heterodimer that is as abundant in the hemolymph, indicating that the observed interactions are specific. None of the proteins we probed for were bound to control beads lacking antibody. Our data suggest that CLIPA2 interacts with

components of the complement pathway, supporting its previously reported role in the regulation of TEP1-mediated immunity (Yassine, *et al.*, 2014).

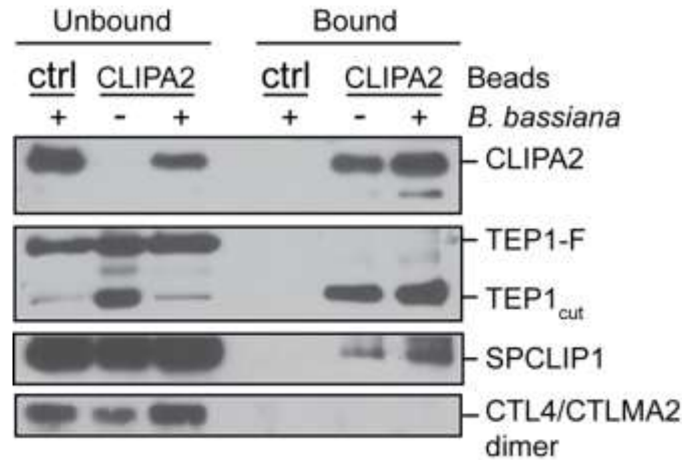


Figure 31. Interaction between CLIPA2 and components of the mosquito complement-like pathway. CLIPA2 cross-linked or control magnetic beads were used in coIP to capture CLIPA2-interacting proteins from the hemolymph of *B. bassiana* infected (+) or naive (-) mosquitoes. Following bead separation and washing, bound and unbound fractions were resolved by non-reducing SDS-PAGE and analyzed by western blot. The blot shown is representative of three independent biological replicates.

The efficiency of αCLIPA2 in IP and the specific interactions observed with TEP1 and SPCLIP1 in our small scale IP assays prompted us to identify the broad spectrum of hemolymph proteins that may interact with CLIPA2. To this aim, CLIPA2 was coIP from hemolymph collected from 900 mosquitoes and followed by the identification of eluant proteins using a highly sensitive nanoLC MS/MS analysis. The list of identified proteins (Table 2) included four members of the CLIPA family (CLIPA1, CLIPA6, CLIPA7 and CLIPA14) but none of these is known to exhibit an RNAi phenotype similar to *CLIPA2* (Volz, *et al.*, 2006 and Osta MA, personal communication). Other proteins in the list included members of the TEP, LRIM, fibrinogen-related proteins, scavenger receptor (SCRASP1) and prophenoloxidase families which are themselves or related family members known to exhibit immune effector roles distinct from that of CLIPA2 including, the killing of *Plasmodium* ookinetes like TEP1 (Blandin, *et al.*, 2004), TEP3 (Povelones, *et al.*, 2011), FBN9

(Dong, *et al.*, 2006), LRIM1 (Povelones, *et al.*, 2009; Osta, *et al.*, 2004) and APL1C (Riehle, *et al.*, 2006), phagocytosis of bacteria like TEP1 and scavenger receptors, (Franc, *et al.*, 1994; Kocks, *et al.*, 2005) and melanization like TEP1 (Blandin, *et al.*, 2004) and PPO (Yassine, *et al.*, 2012; 2014). Hence, these proteins were not further characterized at the genetic level. The remaining proteins on the list with known GO terms are Fondue, a component of the hemolymph clot in *A. gambiae* (Agianian, *et al.*, 2007) and *Drosophila* (Scherfer, *et al.*, 2006); Apolipophorins (Apo) I, II and III which act as lipid transporters; and Gelsolin, a regulator of actin filament assembly. Apo-I and Apo-II are cleavage products of the common precursor protein encoded by the *Apo-II/I* gene (Shapiro, *et al.*, 1988). We were particularly interested in Fondue and the apolipophorins which were also isolated from hemolymph clots indicating their involvement in the coagulation response (Agianian, *et al.*, 2007; Altincicek, *et al.*, 2008; Scherfer, *et al.*, 2004) which, in mammals, is known to cross-talk with the complement system (Amara, *et al.*, 2010). In order to determine whether these proteins exhibit complement regulatory roles similar to CLIPA2, ds*Fondue*, ds*Apo-II/I*, ds*Apo-III*, ds*CLIPA2*, ds*TEP1* and ds*LacZ* (control) mosquitoes were naturally infected with *B. bassiana* and their survival was scored daily. Interestingly, both *Apo-II/I* and *CLIPA2* exhibited similar RNAi phenotypes characterized by increased endurance to fungal infections manifested by significantly better survival rates compared to ds*LacZ* control (Figure 32A), while ds*TEP1* mosquitoes succumbed earlier to the infection as reported previously (Yassine, *et al.*, 2012). On the other hand, ds*Apo-III* and ds*Fondue* mosquitoes exhibited similar survival patterns as ds*LacZ* controls; although ds *Fondue* mosquitoes showed increased endurance to fungal infections in one of the experiments shown, this phenotype however was not reproducible. These results, in addition to the fact that silencing *Apo-II/I* (Mendes, *et al.*, 2008; Rono, *et al.*, 2010; Vlachou, *et al.*,

2005) and *CLIPA2* (Yassine, *et al.*, 2014; Volz, *et al.*, 2006) in *A. gambiae* was shown to reduce the numbers of live *Plasmodium* oocysts in the midgut, raised the possibility that these lipid carriers might be negatively regulating the TEP1-mediated response similar to CLIPA2. Hence, we focused our subsequent functional analysis on *Apo-II/I*.

Table 2. List of proteins identified by mass spectrometry in the CLIPA2 co-IP

Accession	Description	Protein	Score A2	Coverage A2	# Peptides A2	# PSM A2
C9XI64	Thioester-containing protein 1 (Fragment) OS=Anopheles gambiae GN=tep1 PE=4 SV=1 - [C9XI64_ANOGA]	TEP1	236.91	31.46	30	56
Q7PP52	AGAP005888-PA OS=Anopheles gambiae GN=AgaP_AGAP005888 PE=4 SV=2 - [Q7PP52_ANOGA]	charged histidine-rich protein	203.11	47.31	3	54
Q7Q3S5	AGAP008013-PA (Fragment) OS=Anopheles gambiae GN=AgaP_AGAP008013 PE=4 SV=4 - [Q7Q3S5_ANOGA]	orthologue of Dm fondue	109.31	46.92	17	27
E5KED0	AGAP013365-PA OS=Anopheles gambiae GN=Apo3 PE=2 SV=1 - [E5KED0_ANOGA]	apolipoprotein III	94.70	76.68	15	23
F2Y0C4	APL1C OS=Anopheles gambiae GN=APL1C PE=4 SV=1 - [F2Y0C4_ANOGA]	APL1C	85.42	28.40	13	21
Q6VFG8	AGAP011197-RA Fibrinogen (Fragment) OS=Anopheles gambiae GN=FBN9 PE=4 SV=1 - [Q6VFG8_ANOGA]	FBN9	73.65	43.21	13	21
Q7Q398	AGAP007777-PA (Fragment) OS=Anopheles gambiae GN=AgaP_AGAP007777 PE=4 SV=3 - [Q7Q398_ANOGA]	unknown	68.02	28.70	12	17
Q7PUR8	AGAP001826-PA OS=Anopheles gambiae GN=AgaP_AGAP001826 PE=4 SV=5 - [Q7PUR8_ANOGA]	lipophorin ApoI/II	63.33	7.09	18	18
F5HMN2	AGAP013005-PA OS=Anopheles gambiae GN=AgaP_AGAP013005 PE=4 SV=1 - [F5HMN2_ANOGA]	unknown	62.46	35.82	7	15
F5HL03	AGAP013060-PA OS=Anopheles gambiae GN=AgaP_AGAP013060 PE=4 SV=1 - [F5HL03_ANOGA]	unknown	50.39	47.56	6	16
Q7Q7T0	AGAP010816-PA OS=Anopheles gambiae GN=TEP3 PE=4 SV=5 - [Q7Q7T0_ANOGA]	TEP3	48.38	11.57	11	12
Q7PZ88	AGAP011792-PA OS=Anopheles gambiae GN=CLIPA7 PE=3 SV=2 - [Q7PZ88_ANOGA]	CLIPA7	48.06	18.27	10	14
Q7PM31	AGAP009859-PA OS=Anopheles gambiae GN=AgaP_AGAP009859 PE=4 SV=1 - [Q7PM31_ANOGA]	unknown	47.83	33.33	8	15
A7UUU6	AGAP011478-PA OS=Anopheles gambiae GN=AgaP_AGAP011478 PE=4 SV=1 - [A7UUU6_ANOGA]	unknown	45.71	26.62	8	11
Q5TMN0	AGAP011788-PA (Fragment)	CLIPA14	43.38	36.65	6	11

	OS=Anopheles gambiae GN=CLIPA14 PE=3 SV=2 - [Q5TMN0_ANOGA]					
Q7Q4E8	AGAP008364-PA OS=Anopheles gambiae GN=TEP15 PE=4 SV=3 - [Q7Q4E8_ANOGA]	TEP15	41.84	7.66	8	11
Q7PIQ7	AGAP005625-PB OS=Anopheles gambiae GN=SCRASP1 PE=4 SV=3 - [Q7PIQ7_ANOGA]	SCRASP1	41.18	11.91	10	12
A7UVI0	AGAP011604-PA OS=Anopheles gambiae GN=AgaP_AGAP011604 PE=4 SV=1 - [A7UVI0_ANOGA]	unknown	39.84	29.27	4	8
A5A1F9	LRIM1 (Fragment) OS=Anopheles gambiae PE=4 SV=1 - [A5A1F9_ANOGA]	LRIM1	39.11	26.68	11	12
Q7PV63	AGAP011789-PA OS=Anopheles gambiae GN=CLIPA6 PE=3 SV=3 - [Q7PV63_ANOGA]	CLIPA6	36.16	22.25	7	10
F5HLK9	AGAP012966-PA OS=Anopheles gambiae GN=AgaP_AGAP012966 PE=4 SV=1 - [F5HLK9_ANOGA]	unknown	35.20	34.21	4	9
Q7PVY8	AGAP009145-PA (Fragment) OS=Anopheles gambiae GN=AgaP_AGAP009145 PE=4 SV=3 - [Q7PVY8_ANOGA]	unknown	33.04	24.55	5	8
Q7PUI0	AGAP001387-PA OS=Anopheles gambiae GN=AgaP_AGAP001387 PE=4 SV=4 - [Q7PUI0_ANOGA]	unknown	30.77	52.41	4	6
Q7PVY7	AGAP009146-PA OS=Anopheles gambiae GN=AgaP_AGAP009146 PE=4 SV=2 - [Q7PVY7_ANOGA]	unknown	26.99	11.33	5	8
Q7PZ86	AGAP011791-PA OS=Anopheles gambiae GN=CLIPA1 PE=3 SV=2 - [Q7PZ86_ANOGA]	CLIPA1	26.83	25.45	7	8
Q7PV58	AGAP011790-PB OS=Anopheles gambiae GN=CLIPA2 PE=3 SV=4 - [Q7PV58_ANOGA]	CLIPA2	23.55	13.12	5	6
A7UV72	AGAP009106-PA (Fragment) OS=Anopheles gambiae GN=AgaP_AGAP009106 PE=4 SV=1 - [A7UV72_ANOGA]	unknown	21.94	24.11	5	6
Q5TPW1	AGAP011369-PA (Fragment) OS=Anopheles gambiae GN=AgaP_AGAP011369 PE=4 SV=3 - [Q5TPW1_ANOGA]	Gelsolin/RNAi reduces strongly the number of live oocysts(Vlachou)/Gelsolin was found to be part of hemolymph clot in Drosophila	21.66	9.53	6	6
A7URD2	AGAP007453-PA OS=Anopheles gambiae GN=AgaP_AGAP007453 PE=4 SV=1 - [A7URD2_ANOGA]	LRIM9	20.62	10.97	4	6
Q7PTC7	AGAP007663-PA OS=Anopheles gambiae GN=AgaP_AGAP007663 PE=4 SV=2 - [Q7PTC7_ANOGA]	unknown	16.71	14.12	3	5
Q5BN34	AGAP004674-PA OS=Anopheles gambiae GN=AgaP_AGAP004674 PE=2 SV=1 - [Q5BN34_ANOGA]	phenoloxidase inhibitor protein	16.56	16.12	4	4
A0NHB1	AGAP001989-PA OS=Anopheles gambiae GN=AgaP_AGAP001989 PE=4 SV=1 - [A0NHB1_ANOGA]	unknown	16.49	30.74	5	5
O44250	AGAP006258-RA Pro-phenol oxidase subunit 1 OS=Anopheles gambiae PE=2	PPO2	10.68	5.81	3	3

	SV=1 - [O44250_ANOGA]					
B2FZ71	AGAP011239-RA FBN7 protein (Fragment) OS=Anopheles gambiae GN=fbn7 PE=4 SV=1 - [B2FZ71_ANOGA]	FBN7	10.10	19.23	3	3

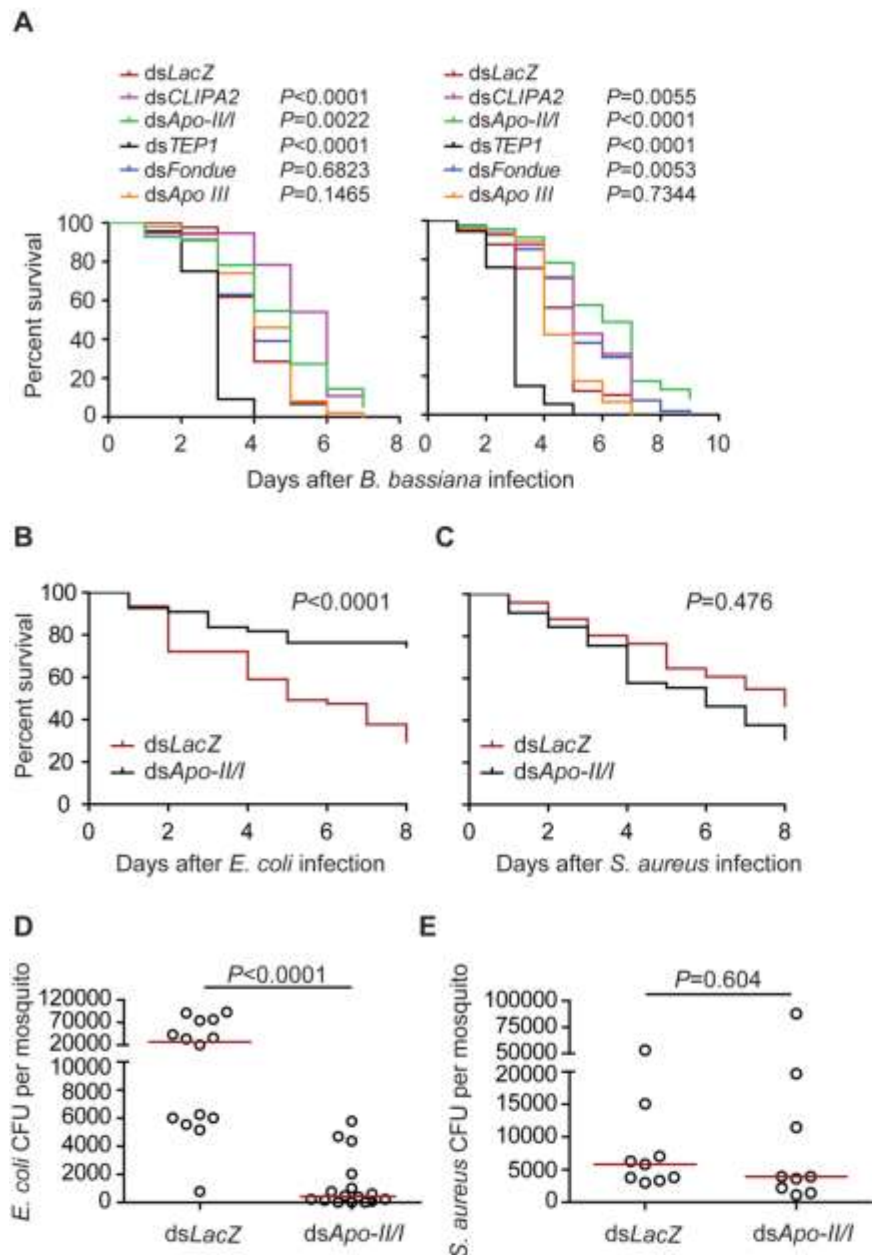


Figure 32. Ds*Apo-II/I* mosquitoes are more resistant to systemic infections with *B. bassiana* and *E. coli*. (A) The indicated mosquito genotypes were sprayed with a suspension of $2x10^8$ spores/ml of *B. bassiana* four days post-dsRNA injection. Dead mosquitoes were counted daily over a period of nine days after fungal challenge. Percent survival was calculated using the Kaplan-Meier method for two independent representative experiments. Statistical significance was calculated using the log rank test. Survival curves were considered to be significantly different if $P < 0.05$. (B-C)

ds*Apo-II/I* mosquitoes were injected with (B) *E. coli* (OD_{600nm} = 0.4) and (C) *S. aureus* (OD_{600nm} = 0.4) and the percent survival was calculated as described in (A). Shown is a representative experiment for each bacterial challenge. (D-E) ds*Apo-II/I* mosquitoes were injected with (D) *E. coli* and (E) *S. aureus* at OD_{600nm} = 0.4. Batches of 8 mosquitoes each were grinded in LB medium at 48 hrs after infection and live bacterial counts (CFUs) were determined by plating serial dilutions on LB plates supplemented with the appropriate antibiotic. Each point on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using the Mann-Whitney test. Medians (red lines) were considered significant if $P < 0.05$. Data shown are from (D) three and (E) two independent biological experiments.

To address whether *Apo-II/I* kd confers resistance also to systemic bacterial infections, like that of CLIPA2 (Yassine, *et al.*, 2014), ds*Apo-II/I* mosquitoes were injected with *E. coli* and *Staphylococcus aureus* and their survival was scored over a period of 8 days. Silencing *Apo-II/I* significantly improved mosquito survival to *E. coli* infections (P<0.0001) relative to ds*LacZ* controls (Figure 32B) and promoted the robust clearance of *E. coli* (P<0.0001) from the hemocoel at 48 hrs post-infection (Figure 32D); the latter phenotype was also observed as early as at 12 hrs post infection in both ds*Apo-II/I* and ds*CLIPA2* mosquitoes (S1 Figure). None of these phenotypes was obtained with *S. aureus* infections (Figure 32C and 32E), suggesting that the enhanced immune response in ds*Apo-II/I* mosquitoes is more efficient in defense against Gram-negative rather than Gram-positive bacteria.

b. <u>The enhanced systemic immune response in *dsApo-II/I* mosquitoes is TEP1-dependent</u>

It was previously reported that TEP1 is required for the increased killing of *Plasmodium* ookinetes in the midgut epithelium of ds*Apo-II/I* mosquitoes (Rono, *et al.*, 2010). To address whether TEP1 is also required for the enhanced immune response to systemic infections in these mosquitoes, we performed an epistatic genetic analysis by RNAi. Interestingly, silencing *TEP1* simultaneously with either *Apo-II/I* or *CLIPA2*

compromised mosquito survival to *B. bassiana* infections as the single *TEP1* kd did (Figure 33A).

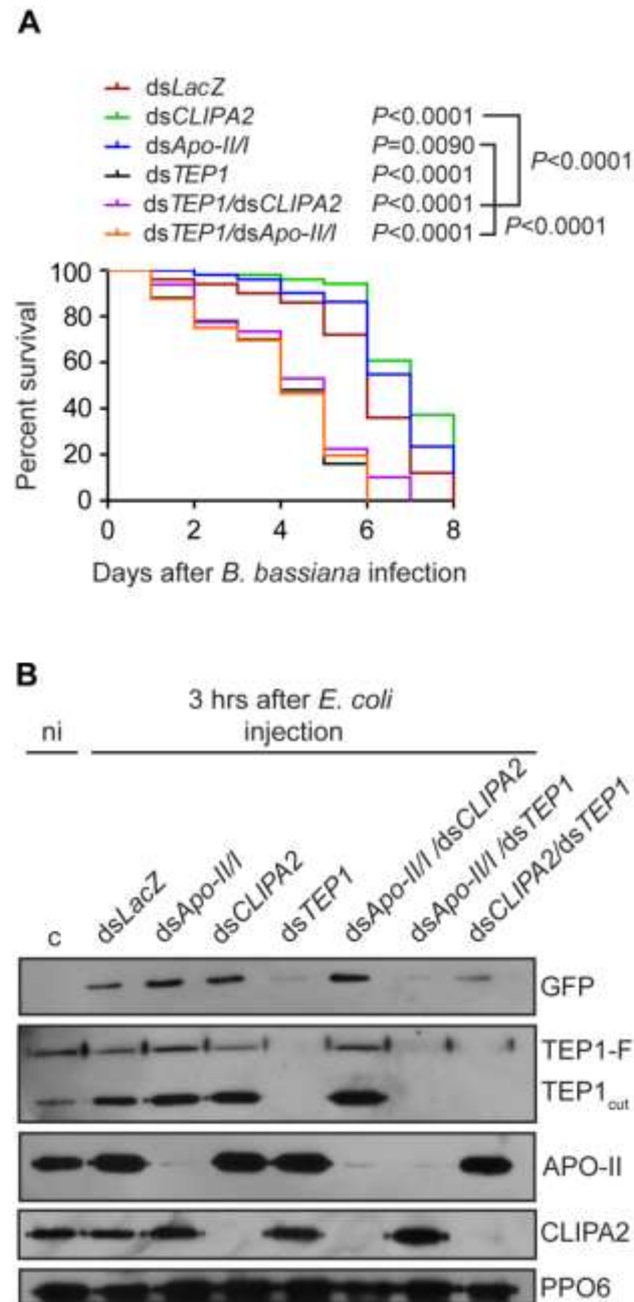


Figure 33. The increased resistance of ds*CLIPA2* and ds*Apo-II/I* mosquitoes to systemic infections is TEP1-dependent. (A) The indicated mosquito genotypes were sprayed with *B. bassiana* spores four days post-dsRNA injection and dead mosquitoes were counted daily after challenge. Percent survival was calculated using the Kaplan-Meier method. Statistical significance was calculated using the log rank test. Survival curves were considered to be significantly different if $P < 0.05$. Data shown are from one representative experiment. (B) Western blot to measure *E. coli* lysis in the hemolymph. Live GFP-expressing *E. coli* were microinjected into the thorax of the indicated mosquito genotypes and hemolymph was extracted 3 hrs later, centrifuged to eliminate cells, and analyzed by western blot using antibodies against GFP, CLIPA2,

Apo-II and TEP1. Protein quantification was performed using the Bradford protein assay and 2 μg of hemolymph proteins were loaded per lane. The blot was also probed with αPPO6 antibody to confirm equal loading. Non-infected (ni) wildtype mosquitoes were used as controls (C). The blot shown is representative of at least two independent biological experiments.

To address whether TEP1 is also required for the increased resistance of ds*Apo-II/I* and ds*CLIPA2* mosquitoes to systemic *E. coli* infections, we designed an assay that could test for TEP1 role and at the same time provide some insight into the mechanism by which TEP1 is mediating the clearance of *E. coli* in these mosquito genotypes. The assay is based on measuring the amount of GFP present in the mosquito hemolymph at 3 hrs after injection of live GFP-expressing *E. coli*. Since these bacteria express cytoplasmic GFP, their lysis is expected to release GFP into the hemolymph which can be then detected by western blot using an anti-GFP antibody. Hence, if TEP1 is indeed engaged in bacterial lysis similar to mammalian complement, then reducing or enhancing TEP1 activity should decrease or increase, respectively, the amount of GFP released from lysed bacteria. Our results revealed a clear GFP band in the hemolymph extract of ds*LacZ* mosquitoes and a very faint band in those silenced for *TEP1* (Figure 33B), suggesting that TEP1 contributes indeed to early bacterial lysis in systemic infections. In ds*Apo-II/I* and ds*CLIPA2* mosquitoes the GFP signal was clearly enhanced relative to ds*LacZ* controls indicating increased bacterial lysis; this increased lysis required TEP1 since the GFP signal in ds*TEP1*/ds*Apo-II/I* and ds*TEP1*/ds*CLIPA2* mosquitoes was again very faint and similar to that in the single *TEP1* kd (Figure 33B). Co-silencing *Apo-II/I* and *CLIPA2* did not enhance the GFP signal over that of the single kd of either gene. Altogether, our data indicate that both CLIPA2 and Apo-II/I negatively regulate the TEP1-mediated immune response and that TEP1 contributes significantly to bacterial lysis in systemic infections. However, this does not exclude

that phagocytosis may be also enhanced in ds*Apo-II/I* and ds*CLIPA2* mosquitoes, despite not being addressed in our assay, since TEP1 is known to act as an opsonin (Levashina, *et al.*, 2001; Moita, *et al.*, 2005).

c. Distinct signaling pathways contribute to CLIPA2 and Apo-II/I upregulation after *B. bassiana* infections

The fact that *Apo-II/I* and *CLIPA2* exhibit similar RNAi phenotypes that are both TEP1-dependent, prompted us to test whether these two genes are similarly regulated during systemic infections in a manner to impose a coordinated control over the complement response. To address this point, we monitored Apo-II, CLIPA2 and TEP1 protein levels in the hemolymph of *A. gambiae* mosquitoes at 48, 72 and 96 hrs after natural infection with *B. bassiana*. Our data revealed an increase in CLIPA2 protein levels in the hemolymph at 48, 72 and 96 hrs after natural infections with *B. bassiana* with the strongest expression detected at the 96 hour time point (Figure 34A). Using real time PCR, we showed that this strong increase at the latter time point correlates with approximately 7 fold increase in CLIPA2 transcript levels (Figure 34B). Apo-II protein levels were also increased at 72 and 96 hrs after *B. bassiana* infections. At the transcript level, the *Apo-II/I* gene exhibited a more modest increase of approximately 2 fold at the 96 hrs time point with respect to non-infected controls; despite showing no statistical significance by two-way ANOVA in this experiment this increase was observed consistently.This is also supported by the clear and reproducible increase in Apo-II proteins in the hemolymph (Figure 34A). Surprisingly, *B. bassiana* did not alter TEP1 protein (Figure 34A) or its transcript levels (Figure 34B) during the course of infection suggesting the existence of a tight control over *TEP1* gene expression during systemic infections. Silencing either of the NF-κB transcription

factors *Rel2* or *Rel1* reduced significantly CLIPA2 transcript (Figure 34B) and protein levels (S2 Figure) after *B. bassiana* infection relative to ds*LacZ* control, indicating that *CLIPA2* gene is regulated by both Toll/Rel1 and Imd/Rel2 immune signaling pathways. Neither *Rel1* nor *Rel2* kd altered significantly *Apo-II/I* expression following infection (Figures 34B and S2) suggesting that it is controlled by another signaling pathway.

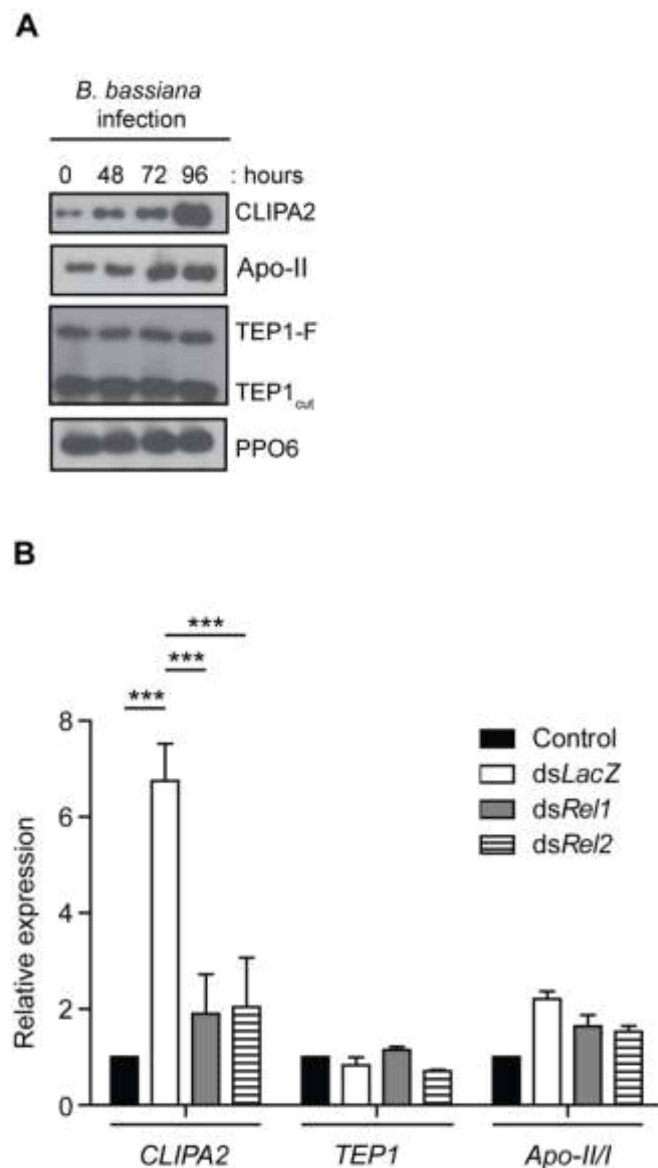


Figure 34. Distinct signaling pathways control the upregulation of *CLIPA2* and *Apo-II/I* following *B. bassiana* infections. (A) A representative western blot showing Apo-II, CLIPA2 and TEP1 proteins levels in hemolymph collected from wildtype mosquitoes at the indicated time points after *B. bassiana* infection. Protein concentration in samples was determined by Bradford protein assay and 0.9 μg of hemolymph proteins were loaded per lane. Additionally, the membrane was probed with αPPO6 to confirm equal loading. (B) Expression levels of *Apo-II/I*, *CLIPA2* and *TEP1*

genes in whole mosquitoes treated with ds*LacZ*, ds*Rel1* or ds*Rel2* at 96 hrs after *B. bassiana* infection. The graph represents the mean expression levels in infected mosquitoes relative to non-infected controls (black bars) for which the mean was adjusted to a value of "1". Data are from three independent biological experiments. Error bars represent SEM. Statistical analysis was performed using two-way ANOVA and means were considered significantly different if $P < 0.05$. ***, $P<0.001$.

d. *Apo-II/I* but not *CLIPA2* kd upregulates *TEP1* expression during systemic infections

CLIPA2 kd does not alter *TEP1* expression following systemic bacterial infections but rather enhances TEP1 protein consumption during immune responses, as previously reported (Yassine, *et al.*, 2014). To address at what level Apo-II/I proteins regulate TEP1 we measured initially the transcript levels of the latter in ds*LacZ*, ds*Apo-II/I* and ds*CLIPA2* naive and *E. coli* infected mosquitoes. Interestingly, TEP1 exhibited a significant 3 fold ($P<0.05$) increase in ds*Apo-II/I* relative to ds*LacZ* mosquitoes at 12 hrs after *E. coli* injection (Figure 35A); whereas, this increase was not observed in naive conditions suggesting that *TEP1* upregulation in ds*Apo-II/I* mosquitoes is infection dependent. In ds*CLIPA2* mosquitoes, *TEP1* expression was similar to that in ds*LacZ* control whether in naive or infected conditions, in agreement with our previous data (Yassine, *et al.*, 2014). We tested in parallel the expression of four AMPs in these genotypes to determine whether other classes of effector molecules are also upregulated after silencing *Apo-II/I*. None of the AMPs tested showed significant change in transcript levels neither in ds*CLIPA2* nor in ds*Apo-II/I* mosquitoes relative to ds*LacZ* control, in both naive and infected conditions (Figure 35A). *CEC1* was strongly upregulated in response to *E. coli* infections; however, to similar levels in ds*LacZ*, ds*CLIPA2* and ds*Apo-II/I* mosquitoes. *DEF1* exhibited a similar expression pattern as *CEC1* but with a more modest upregulation. We then tested whether this enhanced upregulation of *TEP1* in *E. coli*-infected ds*Apo-II/I* mosquitoes is also observed

following *B. bassiana* infections. Indeed, *TEP1* transcripts exhibited a significant 3.2 fold increase (*P*<0.01) in these mosquitoes at 72 hrs post-infection with *B. bassiana* relative to infected ds*LacZ* control (Figure 35B). Again, none of the tested AMPs showed significant changes in gene expression in ds*Apo-II/I* relative to ds*LacZ* after *B. bassiana* infections. TEP1 upregulation was also manifested at the protein level in hemolymph extracts from *B. bassiana* and *E. coli* infected ds*Apo-II/I* mosquitoes (Figure 35C). These data suggest that Apo-II/I proteins negatively control *TEP1* expression during systemic infections, but not in naive conditions. Hence, CLIPA2 and Apo-II/I are acting at different levels to control TEP1-mediated immunity possibly to avoid a cost effect inflicted on the host by an exaggerated systemic immune response.

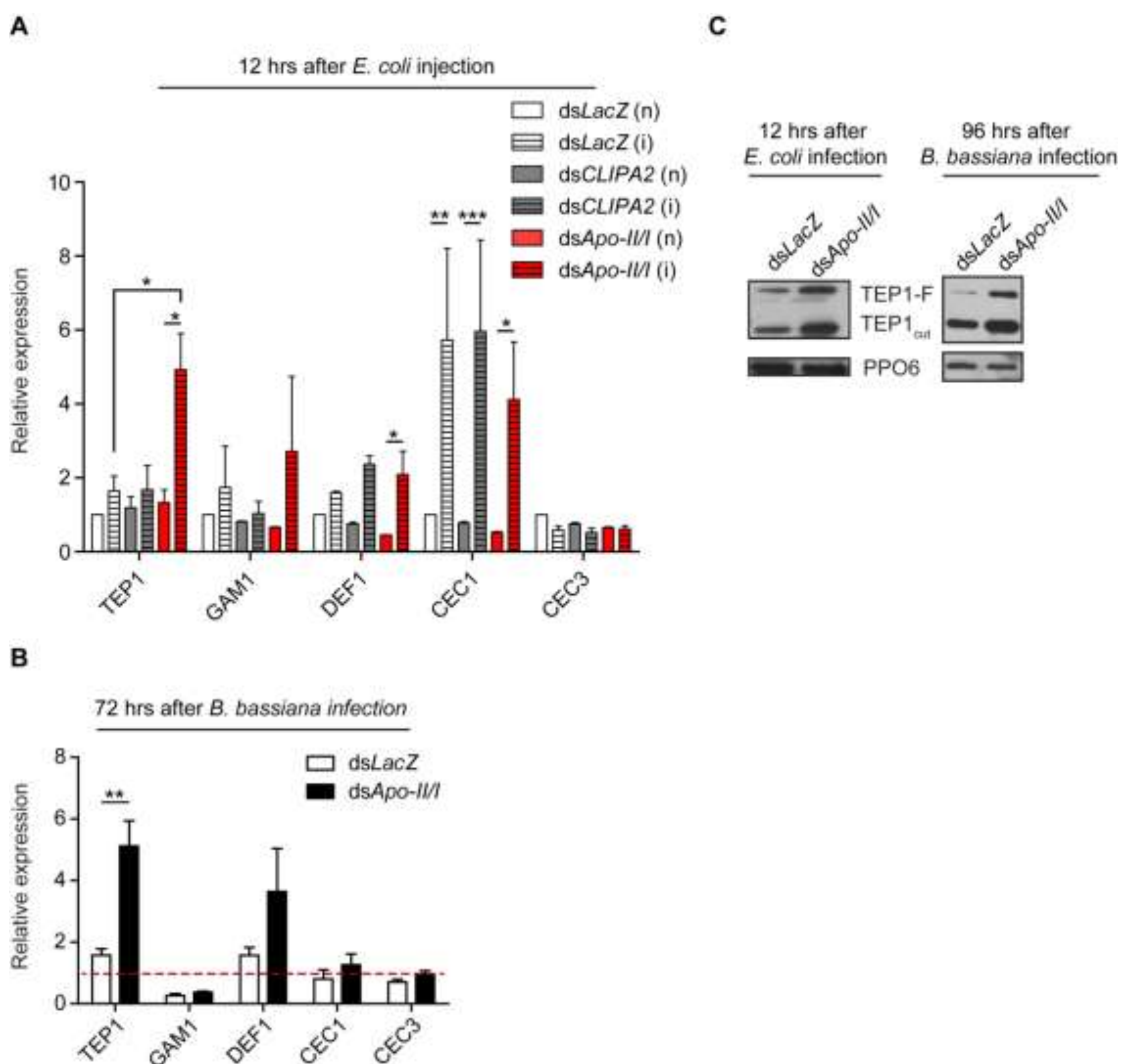


Figure 35. *Apo-II/I* kd upregulates *TEP1* expression during systemic infections. (A) Expression levels of *TEP1, Gambicin (GAM1), Defensin 1 (DEF1), Cecropins 1 (CEC1) and 3 (CEC3)* in ds*LacZ*, ds*CLIPA2* and ds*Apo-II/I* naive (n) or *E. coli* infected (i) mosquitoes. Shown are mean expression levels relative to the ds*LacZ* naive control (plain white bars) for which the mean value was adjusted to a value of "1". Error bars represent SEM of three independent biological experiments. Statistical analysis was performed using two-way ANOVA and means were considered significantly different if $P < 0.05$. *, $P<0.05$; **, $P<0.01$, ***, $P<0.001$. (B) Expression levels of the same genes listed in (A) in ds*LacZ* and ds*Apo II/I* mosquitoes at 72 hrs after infection with *B. bassiana*. Shown are mean expression levels in infected mosquitoes relative to non-infected controls for which the mean was adjusted to a value of "1" indicated by the dashed red line. Error bars represent SEM of three independent biological experiments. Statistical analysis was performed as in (A). (C) A representative western blot showing TEP1 protein levels in ds*LacZ* and ds*Apo-II/I* mosquitoes at the indicated time points after *E. coli* and *B. bassiana* infections. Protein concentrations in hemolymph samples were determined by Bradford protein assay; 0.9 and 0.7 μg of hemolymph proteins were loaded per lane in the *B. bassiana* and *E. coli* infected samples, respectively. Additionally, membranes were probed with αPPO6 to confirm equal loading.

e. <u>*TEP1* upregulation in *B. bassiana* infected ds*Apo-II/I* mosquitoes requires signaling from the JNK pathway</u>

TEP1 gene seems to be subject to complex transcriptional regulation involving Rel1(Frolet, *et al.*, 2006), Rel2 (Frolet, *et al.*, 2006; Garver, *et al.*, 2009; 2011; Guex and Peitsch, 1997) and JNK (Garver, *et al.*, 2013) signaling pathways. Among these, we hypothesized that JNK could be the main pathway responsible for *TEP1* upregulation in ds*Apo-II/I* mosquitoes following systemic infections for the following reasons: first, neither *Rel1* nor *Rel2* kd significantly altered *TEP1* expression at a late time point (96 hrs) during *B. bassiana* infection (Figure 34B); second, the JNK pathway was shown to contribute substantially to *TEP1* basal expression levels in hemocytes (Garver, *et al.*, 2013). To address the role of JNK pathway we questioned whether co-silencing *Apo-II/I* and *Jun* (ds*Jun*/ds*Apo-II/I*), the transcription factor activated downstream of JNK signal transduction pathway (Minden, *et al.*, 1994), abolishes *TEP1* upregulation in response to *B. bassiana* infections. Indeed, while *TEP1* exhibited almost 4 fold upregulation in ds*Apo-II/I* infected relative to ds*Apo-II/I* naive mosquitoes, its expression returned to

basal levels in ds*Jun*/ds*Apo-II/I* infected mosquitoes (Figure 36). No change in *TEP1* expression was observed in ds*LacZ* or ds*Jun* infected mosquitoes relative to naive controls, suggesting that fungal infection itself does not alter *TEP1* transcript levels in wildtype mosquitoes which seem to be maintained at basal levels independent of JNK signaling. On the other hand, fungal infection induced a significant 2.2 fold increase in *Apo-II/I* expression in ds*LacZ* mosquitoes relative to naive controls (Figure 36). This infection-induced upregulation seems to depend on JNK signaling since *Apo-II/I* expression returned to basal levels in infected ds*Jun* mosquitoes.

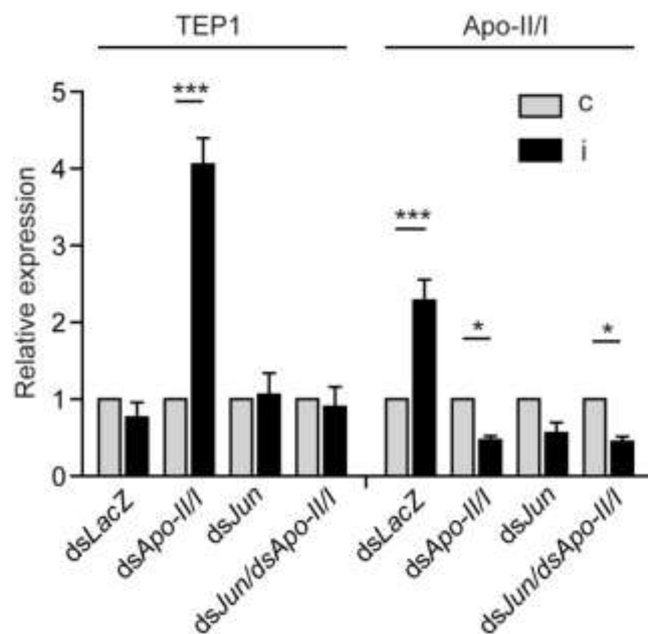


Figure 36. TEP1 upregulation in ds*Apo-II/I* mosquitoes is dependent on the JNK pathway. Expression levels of *TEP1* and *Apo-II/I* in ds*LacZ* (control), ds*Apo-II/I*, ds*Jun* and ds*Jun*/ds*Apo-II/I* mosquitoes at 72 hrs after infection with *B. bassiana*. Shown are the mean expression levels in infected (i) mosquitoes relative to non-infected (c) controls for which the mean was adjusted to a value of "1". Statistical analysis was performed using two-way ANOVA and means were considered significantly different if $P < 0.05$. *, $P<0.05$; **, $P<0.01$, ***, $P<0.001$.

4. Discussion

Insect apolipophorins are complex assemblies of multifunctional molecules that are mainly involved in lipid transport. The lipophorin particle is composed of two obligate proteins Apolipophorin I (Apo-I) and Apolipophorin-II (Apo-II) which are encoded by a single gene *Apo-II/I*, in addition to an exchangeable Apolipophorin-III protein that binds to and stabilizes the interaction of diacylglycerol (DAG) with Apo-I and II forming the low density lipophorins that serve as lipid transport vehicles (Shapiro, *et al.*, 1988). In addition to their classical role in lipid metabolism, several reports pointed to a role for insect lipophorins, particularly Apo-III, in innate immunity. For instance, Apo-III has been involved in microbial recognition (Pratt and Weers, 2004; Whitten, *et al.*, 2004), detoxification of bacterial cell wall components (Halwani, *et al.*, 1997; 2000) and enhancement of humoral (Park, *et al.*, 2005; Wiesner, *et al.*, 1997) and cellular responses (Halwani, *et al.*, 2000; Wiesner, *et al.*, 1997). On the other hand, little information is available on the involvement of Apo-II/I proteins in insect immunity. In *Aedes aegypti*, silencing the *lipophorin* (*Apo-II/I*) gene reduced dramatically the numbers of live *Plasmodium gallinaceum* oocycts in the vector (Cheon, *et al.*, 2006). A similar RNAi phenotype was also reported for *Apo-II/I* in *A. gambiae* infected with *P. berghei* (Rono, *et al.*, 2010; Vlachou, *et al.*, 2005) and *P. falciparum* (Mendes, *et al.*, 2008). Here, we show that silencing *Apo-II/I* renders *A. gambiae* mosquitoes more resistant to systemic infections with *E. coli* and *B. bassiana* by upregulating *TEP1* expression in a JNK pathway dependent manner, providing a mechanistic insight into the enhanced immune phenotype associated with this gene kd. Our data are in agreement with a previous report showing the key role of JNK pathway in controlling *TEP1* expression (Garver, *et al.*, 2013). The RNAi phenotype of *Apo-II/I* suggests that Apo-II/I proteins are likely playing an immune regulatory role during

systemic infections by attenuating or moderating the JNK pathway in mosquito hemocytes, the main, if not, only producers of hemolymph TEP1. Interestingly, anti-inflammatory roles have been also ascribed to certain mammalian apolipoproteins (Sun, *et al.*, 2009; Umemoto, *et al.*, 2013), and in one study Apolipoprotein E was shown to modulate microglial cell inflammation in response to lypopolysachharide treatment by attenuating the JNK pathway (Pocivavsek, *et al.*, 2009).

It remains unclear why silencing *Apo-II/I* triggers this enhanced TEP1-mediated immune response. It is tempting to speculate that *Apo-II/I* kd may be triggering a stress response due to a defect in lipid metabolism. Since Apo-II/I proteins play an essential role in lipid transport, their absence may lead to excess diacylglycerol or triacylglycerol deposition in sites of lipid absorption (midgut) or storage (fat body) while depriving other tissues such as hemocytes, ovaries and flight muscles from these molecules. In both cases, this may lead to endoplasmic reticulum (ER) stress which can increase JNK pathway activity. In fact studies in mammals revealed that JNK activity is increased in response to ER stress (Urano, *et al.*, 2000) and to excessive accumulation of lipids in tissue (Fu, *et al.*, 2011; Ozcan, *et al.*, 2004). Alternatively, we cannot exclude that apolipophorins may moderate the JNK pathway through bioactive lipids they might carry. A recent study in *A. gambiae* revealed that a lipid carrier protein of the lipocalin family carries the lipid lipoxin that acts as a hemocyte differentiation factor in the mosquito *A. gambiae* (Ramirez, *et al.*, 2015), in a manner dependent on the Toll, STAT and JNK pathways (Ramirez, *et al.*, 2014). Future biochemical analysis of the composition of these apolipophorin particles in infected mosquitoes may provide new insight into their functional interaction with the immune system. Rono *et al*, have previously shown that the reduction in the number of *P. berghei* oocysts in *Apo-II/I* kd mosquitoes is TEP1-dependent, however *TEP1* expression was not altered in these

mosquitoes relative to ds*LacZ* controls (Rono, *et al.*, 2010), in contrast to the robust upregulation observed in our systemic infections of ds*Apo-II/I* mosquitoes. A plausible explanation to this apparent discrepancy is that during systemic infections the microbes in the hemolymph may deliver stronger immune stimulatory signals to hemocytes, the main producers of TEP1, than ookinetes which are physically separated from hemocytes by the midgut epithelium. In other words, the robust *TEP1* upregulation in ds*Apo-II/I* mosquitoes during systemic infections may require, in addition to JNK, contribution from other signaling pathways that may not be stimulated in hemocytes during *Plasmodium* midgut invasion. The identity of these pathways and the molecular basis of moderation of the JNK pathway by these apolipophorins remain to be determined.

It was interesting to note that *E. coli* and *B. bassiana* infections in ds*LacZ* mosquitoes did not trigger any significant upregulation of *TEP1* compared to ds*LacZ* naive controls. Our results are in agreement with the study of Baton, 2009 in which *TEP1* expression was not altered in the hemocyte transcriptome of wildtype *A. gambiae* infected with *E. coli* and *Micrococcus luteus*, suggesting that a tight control over TEP1 expression is imposed during systemic infections possibly to avoid an exaggerated immune response that may be costly to the host. Such cost was previously reported in ds*CLIPA2* mosquitoes in the form of compromised female fecundity (Yassine, *et al.*, 2014).

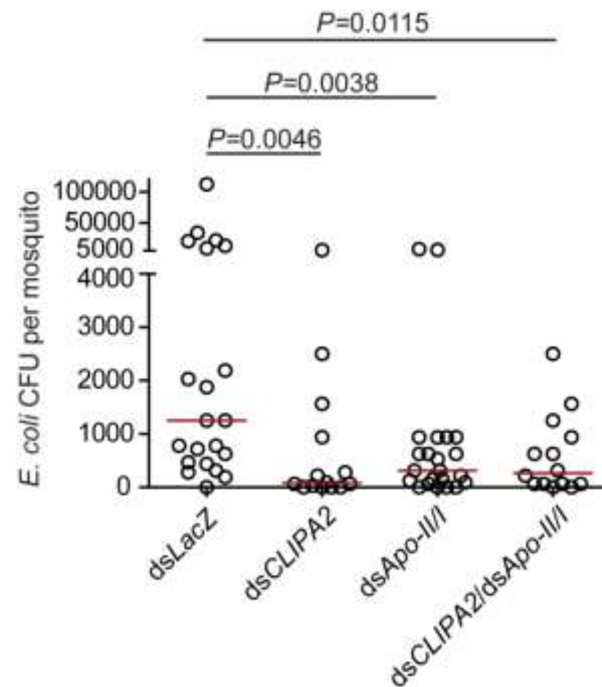
While *dsApo-II/I* mosquitoes exhibited enhanced resistance to *E. coli* infections, their resistance to *S. aureus* was similar to controls despite the robust *TEP1* upregulation, suggesting that mosquito complement is more effective in dealing with Gram-negative than Gram-positive bacteria. These results may seem intriguing because TEP1 was shown to be required for the phagocytosis of both *E. coli* and *S. aureus in vivo* (Moita, *et al.*, 2005); however, they also suggest that mosquito complement elicits additional effector responses that are more effective in killing Gram-negative (such as lysis) than

Gram-positive bacteria. We have shown that TEP1 contributes significantly to *E. coli* lysis; however, we were not able to address whether it has the same effect on *S. aureus* since we lack a GFP-expressing strain. Nevertheless, it won't be unexpected for *S. aureus* particularly to resist TEP1-mediated lysis, since the thick peptidoglycan layer surrounding these Gram-positive bacteria is one of the arsenals they use to escape lysis by mammalian complement (Frank, 2001; Lambris, *et al.*, 2008). Also, the fact that the gut microbiome of field caught *A. gambiae* mosquitoes is composed largely of Gram-negative bacterial species (Boissiere, *et al.*, 2012), and since systemic bacterial infections in the field are likely to occur mainly through gut invasion, indicate that mosquito complement may have evolved to deal more efficiently with Gram-negative bacteria.

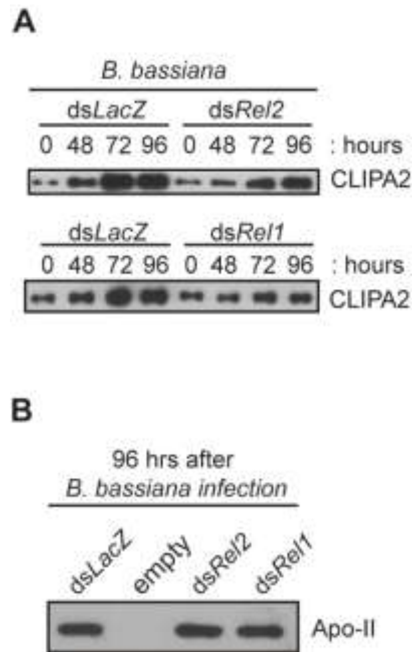
The identification of Apolipophorins among the proteins that co-immunoprecipitated with CLIPA2 was a bit surprising but may support early observations that the lipid particles nucleated by lipid carrier proteins in the hemolymph may in fact serve as platforms for recruiting immune as well as other proteins. Indeed, immune proteins including phenoloxidase and pattern recognition receptors were found associated with lipid particles isolated from the hemolymph of *Ephestia Kuehniella* (Rahman, *et al.*, 2006) and in *Drosophila* larvae, apolipophorins were shown to carry secreted signaling molecules that act as tissue morphogens (Panakova, *et al.*, 2005). We have shown using the bioparticle surface extraction assay that Apo-II binds to the surface of *E. coli* bioparticles in a TEP1 independent manner (S3 Figure). This could indicate that Apo-II/I proteins may either shuttle immune proteins to microbial surfaces possibly by interacting with lipids exposed on these surfaces, or contribute to microbial clearance, or detoxify immune-stimulatory cell wall components as has been ascribed to Apo-III. Identifying the molecular composition of lipophorin particles from mosquitoes

coupled with genome-wide gene expression studies in *Apo-II/I* kd genotypes challenged with different microbes is expected to reveal novel insight into the mechanisms that link lipid metabolism to immune gene regulation.

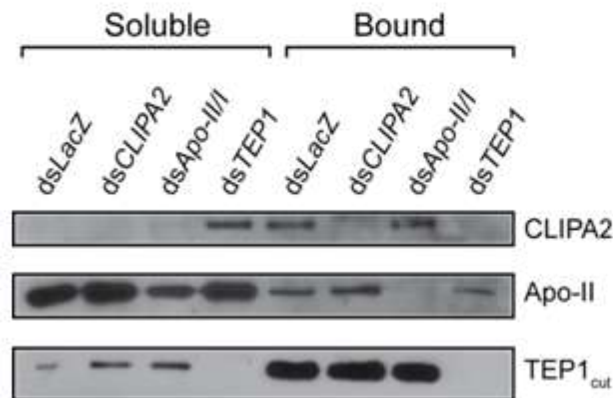
Supplementary figures



S1. Early clearance of injected *E. coli* in ds*Apo-II/I* mosquitoes. Ds*Apo-II/I*, ds*CLIPA2,* ds*CLIPA2*/ds*Apo-II/I* and ds*LacZ* (control) treated mosquitoes were injected with *E. coli* at OD_{600nm} = 0.4. Batches of 8 mosquitoes each were grinded in LB medium at 12 hrs after bacterial injection and live bacterial counts (CFUs) were determined by plating serial dilutions on LB plates supplemented with the appropriate antibiotic. Each point on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using the Mann-Whitney test. Medians (red lines) were considered significant if $P < 0.05$. Data shown are from four independent biological experiments.



S2.Western blot showing CLIPA2 hemolymph proteins levels in ds*LacZ*, ds*Rel1* and ds*Rel2* mosquitoes at the indicated time points after *B. bassiana* infection. Hemolymph proteins were quantified by Bradford protein assay and 0.7 ug were loaded per lane from each treatment. (B) Western blot showing Apo-II protein levels in ds*LacZ*, ds*Rel1* and ds*Rel2* mosquitoes at 96 hrs after *B. bassiana* infection. Hemolymph proteins were quantified by Bradford protein assay and 0.56 µg of proteins was loaded per lane from each treatment.



S3. Apo-II/I proteins are recruited to bacterial surfaces. *E. coli* bioparticles were microinjected into the thorax of the indicated mosquito genotypes and hemolymph was extracted 20 min later. Bioparticles were pelleted by centrifugation, and the soluble fractions collected. Bacterial pellets were washed with buffer before bound proteins were extracted. Soluble and bound fractions were resolved by non-reducing SDS-PAGE and analyzed by western blot using antibodies against CLIPA2, Apo-II and TEP1. The image is representative of two independent experiments.

CHAPTER V

GENERAL DISCUSSION

Mosquito immune responses to natural fungal infections remain poorly understood despite the ubiquitous nature of these microorganisms and their route of infection that bypasses the midgut in contrast to infections with bacteria and malaria parasites. Hence, most of this thesis work focused on utilizing the entomopathogenic fungus *B. bassiana* as a model organism in order to characterize, at the functional and molecular levels, the specific roles of key immunity genes, in particular clip-SPHs, in immune defense. The contribution of certain immune effector responses to anti-fungal defense have been also addressed. What prompted the use of fungi, and in certain cases bacteria, in mosquito infections rather than malaria parasites is the increasing belief in the field that the evolutionary forces that have shaped mosquito immunity are more likely attributed to bacterial and fungal populations present in the larval habitats, rather than to infections with malaria parasites (White, *et al.,* 2011).This is supported by the observation that the composition of the bacterial flora in adult mosquito midguts significantly influences the development of *Plasmodium* parasites in the vector either indirectly through a process known as immune priming or directly through bacterial secreted products toxic to the parasite (Boissiere, *et al.,* 2012; Cirimotich, *et al.,* 2011a, 2011b ;Dong, *et al.,* 2009 ;Meister, *et al.,* 2009). Despite the fact that the gut microbiota is likely to include also yeast and fungi, the contribution of these to vector competence remain to be elucidated. Interestingly, there has been a report on the pathogenicity of Coelomomyces fungi to *A. gambiae* larvae in aquatic habitats (Muspratt, 1946), suggesting that these microorganisms are likely to impose a substantial pressure on the mosquito immune response.

In order to use fungi as model organisms to challenge mosquitoes, we had to design a tractable method of infection that mimics as much as possible the mode of infection occurring in the field. Field mosquitoes acquire fungal spores through tarsal contact by landing on surfaces contaminated with these spores. To mimic this process we anaesthetized mosquitoes by brief exposure to low temperature of 4°C, then sprayed them with a suspension of *B. bassiana* spores placed in fragrant sprayers. This gentle method of infection is not harsh on the insect and its sensitivity and reliability was tested by comparing the susceptibility of adult female *A. gambiae* mosquitoes to two strains of *B. bassiana*; a wildtype strain and a transgenic strain expressing *Aedes aegypti* TMOF (*Bb*-Aa1) that interferes with mosquito digestion by blocking the expression of trypsin (Borovsky, *et al.,* 2006). Also, TMOF was shown to be resistant to proteolysis in the mosquito gut and could easily bypass the gut and reach the hemolymph of both larvae and adults. If fed to mosquitoes, TMOF could disrupt food digestion, cause anorexia, and result in the death of the mosquito (Borovsky, *et al.,* 1993; Borovsky and Meola, 2004; Borovsky 2003; Bylemans, *et al.,* 1994). Our results showed that *Bb*-Aa1 infection was more potent and virulent in both sugar and blood fed adult female *Anopheles* as compared to those infected with the wild-type fungal strain. Around 40% reduction in the LD50 values (50% mortality) was scored in both groups compared to the wild type and around 15 and 25% reduction in the mean survival times (LT50 values) of sugar and blood fed groups, respectively, as compared to the wild type control strain. Similarly, *Bb*-Aa1 infected *A. gambiae* larvae exhibited a 20 and 27% decrease in the LD50 and LT50 values, respectively, as compared to the wild type control strain. *Bb*-Aa1 infected larvae also showed severe compromised-retarded growth evidenced by their abnormally small size compared to WT infected groups (bigger in size) and non-infected control groups (normal size). At the level of

reproduction, both fungal strains affected egg laying in infected mosquitoes; yet, a significant reduction in the number of eggs produced in *Bb*-Aa1 infected adults (60% less eggs) was recorded as compared to WT infected ones (16%). Collectively, these data suggest that our infection model is reliable as it clearly distinguished between the pathogenicity levels of the two *B. bassiana* strains and hence, could be used in future studies to dissect mosquito immune responses to fungal infections. As such, we took advantage of this robust method of infecting mosquitoes with fungi to address the contribution of melanization to anti-fungal defense in *A. gambiae* mosquitoes. Melanization, a potent immune response unique to arthropods, is characterized by the deposition of a dense melanin coat around the invading pathogen leading to its physical entrapment and eventual death by asphyxiation, or around a wounded area resulting in wound healing. Despite the fact that the melanization response has been well studied in several model insects (Ashida, et al., 1990; Reviewed in Christensen, *et al.,* 2005; Jiang, *et al.,* 2011; Volz, *et al.,* 2006), its role in insect immunity is still controversial as some believe that the primary role of melanization is wound healing. In *A.gambiae* for example, melanization was not required for the mosquito survival against infections with *E. coli* and *S. aureus* (Schnitger, *et al.,* 2007). However, since these two bacteria are not natural pathogens of mosquitoes in the field the results of this study may not necessarily exclude the importance of melanization against specific bacterial pathogens. Moreover, melanization does not seem to be induced by *Plasmodium* parasites in field caught mosquitoes (Niare, *et al.,* 2002). Here, we showed that melanization plays indeed an important role in anti-fungal immunity, significantly retarding the growth of *B. bassiana*; silencing CLIPA8, a key positive regulator of the melanization response, compromised mosquito survival after fungal infections. We have observed a dense melanin coat around hyphal filaments in dissected mosquito tissues indicating that fungi

are strong activators of that response. Additionally, TEP1 exhibited a similar but relatively stronger RNAi phenotype than CLIPA8 indicating that mosquito complement also contributes to anti-fungal defense. Altogether, our results inform a pathogen-specific role for melanization in mosquito immunity. The fact that this response is not triggered by *Plasmodium* parasites suggests that these parasites evolved mechanisms to avoid that response or that they are quickly and efficiently eliminated by other effector responses such as lysis. It is worth mentioning here that one key distinction between *Plasmodium* ookinetes (the parasite stages that are melanized in certain mosquito genotypes generated in the lab) on one hand and bacteria and fungi on the other hand is that the latter group have much thicker and elaborate cell walls that might resist lysis in certain instances. A recent study revealed that the human malaria parasite utilizes a surface protein to avoid mosquito complement activation which is required to activate the melanization response suggesting active suppression of the host immune response (Molina-Cruz, *et al.,* 2013). Future studies should examine a broad panel of bacteria and fungi that are relevant to mosquito larval habitats to see whether certain species that are often in contact with mosquitoes are able to escape or suppress some mosquito immune responses.

In the third study, I mainly focused on characterizing the role of CLIPA2, an SPH that was previously reported to act as a negative regulator of *Plasmodium* ookinete melanization in *A. gambiae* (Volz, *et al.,* 2006), in mosquito immune responses to systemic fungal and bacterial infections. Our results revealed that CLIPA2 contributes to mosquito tolerance and resistance to bacterial and fungal infections by acting as a negative regulator of the complement protein TEP-1. Silencing CLIPA2 significantly enhanced TEP1 consumption and consequently TEP1-mediated immune reactions such as melanization and lysis. However, this exaggerated immune response triggered in

CLIPA2 kd mosquitoes was associated with a reduction in egg laying, suggesting the existence of a trade-off between immunity and reproduction. An interesting outcome of this study is that it confirmed the tight functional interaction between the complement system and the melanization response (or phenoloxidase activation). Previous studies have already highlighted the requirement of TEP1 in the melanization response (Blandin, *et al.,* 2004; Yassine, *et al.,* 2012). Here we showed that increasing TEP1 activity enhanced hemolymph PO activity further indicating the tight link between both systems. One key question that remains to be answered is in what context and how TEP1 triggers PO activation. Concerning the first part of the question it is clear that not every time TEP1 is active PO becomes activated. This is supported by the fact that TEP1 is activated during infections with *Plasmodium* parasites yet PO is not. Whereas during bacterial and fungal systemic infections TEP1 activation leads to the activation of PO. A possible explanation to this phenomenon is that the melanization response may require a threshold concentration of active TEP1 ($TEP1_{cut}$) on microbial surfaces to be initiated. The fact that *Plasmodium* ookinetes are simple eukaryotic cells lacking additional cell walls may render them easy targets for TEP1-mediated lysis and hence are killed before $TEP1_{cut}$ reaches the threshold concentration needed to activate PO. Whereas such concentrations may be achieved on microbial cells with more resistant cell walls such as bacteria and fungi. In *Holotrichia diomphalia* for example, a serine protease homolog was shown to cluster with PPO in a 1:1 ratio forming a megadalton complex (Piao, *et al.,* 2005), suggesting that efficient PPO activation might require the formation of substantially large clusters on target surfaces. To answer the second part of the question it is imperative to understand the mechanisms that recruit PO to microbial surfaces to initiate the biosynthesis of melanin and how these mechanisms allow the distinction between microbial surfaces and those of host cells. It is clear that this is a

complex process and entails additional factors than a simple PRR guided delivery process. Future studies coupling proteomics and advanced microscopy are likely to provide new molecular details into these processes; since to date not a single PRR have been identified in the mosquito that inhibits the melanization response.

In order to identify additional complement regulators and in the aim of providing new insight into CLIPA2 function, we co-immunoprecipitated CLIPA2 from the hemolymph of *B. bassiana* infected mosquitoes and identified the proteins that coIP with CLIPA2 by mass spectrometry. Interestingly TEP1 was the top hit on the list, providing support to the role of CLIPA2 in TEP1 regulation. We also showed by western blot that SPCLIP1 coIP with CLIPA2 indicating that these CLIPs are probably part of a large protein complex including TEP1 and possibly other proteins. Functional genetic analysis of selected proteins that coIP with CLIPA2 showed that *Apolipophorin-II/I* (*Apo-II/I*) gene, encoding the two lipid carrier proteins Apo-I and II, exhibits a similar RNAi phenotype as CLIPA2. Both *Apo-II/I* kd and *CLIPA2* kd enhanced mosquito resistance to *B. bassiana* and *E. coli* infections in a TEP1-dependent manner. Interestingly, *Apo-II/I* kd triggered a significant up-regulation in *TEP1* mRNA levels in a c-Jun N-terminal kinase (JNK) pathway-dependent manner indicating a role for Apo-II/I in controlling the JNK pathway during systemic infections. As such, it seems that CLIPA2 and Apo-II/I function through different TEP1 control mechanisms. While CLIPA2 seems to be mainly involved in tightly regulating TEP1 consumption, Apo-II/I seems to be involved in regulating *TEP1* at the mRNA levels. As such, we believe that Apo-II/I is playing a regulatory role to attenuate or moderate the activation of the JNK pathway in infected mosquitoes. The fact that Apo-II/I coIP with CLIPA2 suggests that these apolipophorin particles may constitute platforms for the assembly of immune protein complexes. In fact, Apo-II/I have been also identified among the proteins that

co-IP with the lectin complex CTL4/CTLMA2 (Christophides and Osta, unpublished). It remains to be determined whether the co-purifying proteins detected using pull down assays represent true partners in *bona fide* protein complexes. Future studies investigating the composition and dynamics of protein complexes in the mosquito hemolymph will require the use of native protein separation techniques such as size-exclusion chromatography coupled with mass spectrometry. However, using these approaches in the mosquito remains a challenge due to limitations in sample size.

In summary, our results highlight the complexity of complement regulation in the mosquito and reveal the existence of functional cross-talk between the immune and metabolic systems in the insect. They also raise several questions that remain to be answered. For instance, what targets mosquito complement to microbial surfaces? Is there a role for pattern recognition receptors in that process as in the mammalian system? How does complement activation regulate downstream effector responses including lysis, melanization and phagocytosis and does this vary with the nature of the microbe? Coupling advanced microscopy with proteomic and functional genetic studies will be essential to address these questions in the future. Complementing such studies in the vector with functional genetic screens in the malaria parasite may reveal novel targets to eventually block the transmission of the malaria parasite by the mosquito vector.

CHAPTER VI

MATERIALS AND METHODS

A. Ethics statement

All the conducted work was done in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, U.S.A. The Animal protocol was approved by the Institute Animal Care and Use committee (IACUC) (permit number 11-09-199), which works in agreement with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals (USA), and abides by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, U.S.A.

B. Materials

1. Anopheles gambiae mosquito strain

The G3 *Anopheles gambiae* mosquito strain was used in all experiments. Mosquitoes were reared at 27°C (± 1°C), 80% humidity, in a 12h day-night cycle. Larvae and adult mosquitoes were fed tropical fish food and 10% sucrose, respectively. For egg production, adults were given a blood meal by allowing them to feed on an anaesthetized Balb/c mouse. Mice were anaesthetized by intraperitoneal injection of a 100µl mixed Ketamine/Xylazine solution, containing 4.2mg of Ketamine and 0.3mg of Xylazine.

2. Bacterial, fungal, and P.berghei parasite strains

Ampicillin resistant GFP-expressing *E.coli* bacterial strain OP-50 (a kind gift from J.J. Ewbank) and tetracycline resistant *S.aureus* (a kind gift from P. Bulet)

were grown on Luria-Bertani (LB) plates supplemented with 100 μg/ml ampicillin and 50 μg/ml tetracyclin antibiotics, respectively.

GFP-expressing (strain 242-GFP) *B .bassiana* (a kind gift from M. Bidochka), Wild-type *B .bassiana* (80.2) (a kind gift from D. Ferrandon), and transgenic *B. bassiana* expressing *Aedes aegypti* TMOF (strain *Bb*-Aa1) (generated in N. Keyhani's lab) fungal strains were grown on potato dextrose agar (PDA) plates, in a 75-80% humid environment, at 25°C, in the dark. For spore extraction, 10ml ddH_2O was added to each 3-4 weeks old fungal plates, and a sterile cell scraper was used to scrap the surface of mycelia and collect the fungal conidia (spores). A sterile funnel containing autoclaved glass wool was used to separate the spores from other mycelia structures. The collected spore suspension was washed twice with ddH_2O, centrifugated at 4000 rpm, counted and diluted to the desired concentration. Freshly prepared fungal spore solutions were used for all mosquito challenges.

GFP-expressing *P.berghei* parasite strain (CON_{GFP}) (Franke-Fayard, *et al.,* 2004) was passaged in Balb/c mice. Blood smears were prepared from a drop of blood obtained from the mouse tail and stained using Diff-Quick staining kit to determine the percentage of parasitemia. Mosquitoes were infected with the GFP-expressing *P. berghei* parasite by feeding on mice having around 8-10% parasitemia. All feeding on *P. berghei*-infected mice were carried in an incubator set at 19°C.

3. Eukaryotic insect cell line

Sf9 mosquito cells (Invitrogen) were grown in a serum-free Sf-900 II SFM medium (Invitrogen) containing streptomycin (50μg/μl) and penicillin (50U/μL). In cases where antibiotic-antimycotic solution (10,000 U/mL of penicillin, 10,000 μg/mL

of streptomycin, and 25 µg/mL Fungizone) was also added to the media, the aforementioned concentration of streptomycin and penicillin were reduced by half.

C. Mosquito bioassays and quantification of microbial proliferation

1. Mosquito infections

a. Bacterial infections

GFP-expressing-Ampicillin resistant *E. coli* culture was grown overnight at 37°C, pelleted by centrifugation, and washed four-five times with 1xPBS. The bacterial pellet was then re-suspended in 1xPBS, and adjusted to O.D. $_{600nm}$ of 0.4. Individual female mosquitoes having *LacZ* kd, *TEP1* kd, *CLIPA2* kd, or *Lp* kd were injected four days post silencing with 69 nL of the prepared bacterial culture. For the survival bioassays, mosquito survival/death was scored every day, for one week. Three independent biological repeats were performed for each experiment. The percent survival of each experiment was calculated using The Kaplan-Meier survival test and the Log-rank test was used to calculate the statistical significance of the observed differences. A p-value <0.05 was considered significant. For the *E. coli* proliferation assay, 4-5 batches of 7 mosquitoes per genotype were grinded on ice in 400 µl 1xPBS. The homogenates were then plated in a serial dilution manner (1:1000, 1:10000, 1:100000) on LB agar plates containing Ampicillin, and placed at 37°C O.N. The number of bacterial colony forming units per mosquito was counted. The experiment was repeated three independent times. The Mann-Whitney test was used to calculate the statistical significance of the scored differences between all tested genotypes. A p-value < 0.05 indicated significant mean differences.

For bioparticle bacterial assays, around 60 female mosquitoes were injected with a 20mg/ml bacterial bioparticle suspension of fluorescein or pHrodo labeled E.coli

K-12 bacterial strain (Invitrogen) re-suspended in 1x sterile PBS (around 4×10^5 bacteria/69nl). Mosquito hemolymph was extracted 30 minutes after bioparticle injection into non-reducing SDS-PAGE sample buffer and analyzed using western blot as previously described.

b. Fungal infections

For mosquito fungal infection assays, two different modes of infection were used; either by natural infection or through spore injection. For the survival experiments, a group of fifty to sixty female mosquitoes per genotype were injected with dsRNA for *LacZ* kd, *TEP1*kd, *CLIPA2* kd, *CLIPA8* kd, *Lp* kd, *APOIII* kd, or *Fondue* kd. Three-four days post silencing, mosquitoes were anaesthetized on ice and either sprayed with WT fungal spore solution (strain 80.2) of 10×10^7 spore/ml concentration prepared in 0.05% Tween-80, placed in a glass atomizer obtained from sally@Accessories for Fragrances.com, or gently dragged over a PDA fungal spore plate. The mosquito survival rates were scored every day, for one week. In experiments where *Anopheles* susceptibility to different fungal strains was tested, a transgenic *Beauveria bassiana* strain expressing *Aedes aegypti* TMOF (*Bb*-Aa1) was used in addition to the wild type *Beauveria bassiana* (strain 80.2). Adult mosquitoes (both sugar fed and blood fed) were sprayed with different fungal concentrations ranging from 1×10^7 to 2×10^8 conidia/ml, as previously described. Larval survival bioassays were, in turn, conducted in plastic trays ($8.5 \times 13 \times 5.5$cm in dimension), containing around 40 mosquito larvae (three-days old) in a total of 110 ml water. Larvae were infected by applying different concentrations of fungal spores ranging 9×10^5 to 1×10^7conidia/ml, on a 50 µl olive oil suspension placed on the surface of the plastic trays, and mixed horizontally back and forth to spread the spores homogenously on the

surface of the plastic tray. Three independent biological repeats were performed at least for each experiment using different batches of mosquitoes and spores. The percent survival over an indicated time scale (in days) was calculated using The Kaplan-Meier survival test, and the Log-rank test was used to calculate the statistical significance of the observed differences. A p-value <0.05 was considered significant. The regression analysis test, using the survival curves, was used to calculate the Median lethal time (LT50) values of WT and TMOF *B. bassiana* infected adult *Anopheles* (sugar and blood fed) and larvae infected with 1×10^8 and 5×10^6 conidia/ml, respectively. The Student's t-test was used to calculate the statistical analysis of LT50. The Probit analysis was used to determine the Median lethal dose (LD50) using concentrations ranging from 1×10^7 to 2×10^8 conidia/ml in adult mosquitoes and 9×10^5 to 1×10^7 conidia/ml in larvae.

For fungal proliferation bioassays, *LacZkd*, *TEP1kd*, and *CLIPA8kd Anopheles* were sprayed with a concentration of 5×10^7 spores/ml of a WT *B. bassiana* fungal solution (strain 80.2) as previously mentioned. Ninety six hours post infection; around 15 mosquito batches per genotype, with 2 females in each batch, were grinded in 400µl double distilled water containing 0.05% Tween-80. The homogenate was properly vortexed, and 50 µl of it were plated on selective PDA plates containing in addition to PDA, 250µg/ml dodine (Sigma), 1mg/mL yeast extract, a cocktail of antibiotics including: 50µg/ml streptomycin, 50µg/ml penicillin, 50µg/mL gentamycin, and 5µg/mL crystal violet. The fungal plates were incubated at 27°C in 70-80% humidity. The hyphal body colony forming units were counted six days after performing the experiment, and the results of two independent biological repeats were taken. The Mann-Whitney test was used to calculate the statistical significance between the different tested groups. A p-value <0.05 indicated significant difference among means.

In few experiments, mosquitoes were maintained on antibiotics to rule out the possible influence of the microbial flora on the obtained results. In brief, freshly collected adults were fed on 10% sucrose containing antibiotic mix of gentamycin (15μg/mL), penicillin (10U/mL) and streptomycin (10μg/mL) for six days prior to the fungal infection assays. Antibiotic treated sugar pads were changed every 12h to make sure that the supplied antibiotic remains always fresh. The antibiotic regime was continued for one more day post fungal infection, then stopped.

c. *P. berghei* parasite infection

Three to four days post gene silencing, mosquitoes were fed on an anaesthetized-*P.berghei* infected mouse with around 8% parasitemia. Infected mosquitoes were placed in a humid container, in a 12 h day-night cycle incubator, at 19–21°C. 24h post infection, mosquitoes were anaesthetized on CO2, and non-fed mosquitoes were removed. 7-9 days post infection, infected mosquito midguts were dissected, fixed for 50min in 4% paraformaldehyde, washed with 3x with 1x PBS (10 minutes each wash), and mounted in Vectashield mounting medium (Vector Laboratories). Fluorescence microscope was used to score for live GFP-expressing oocycts and melanized ookinetes enclosed in a dark brown melanin capsule. The Mann-Whitney test was used to calculate the statistical significance between the different tested groups.

D. Molecular biology

1. Gene knockdown by RNA interference

Genes of interest were silenced in adult female mosquitoes by the micro-injection of double stranded RNA (dsRNA) into the thorax as previously described (Blandin, *et al.,* 2002). For dsRNA production, gene specific DNA amplicons flanked

by T7 promoter sequences were produced by PCR amplification of mosquito cDNA or plasmids containing full length cDNA sequence (95°C for 3min; 95°C for 45s; 60°C for 1min; 72°C for 1min; cycles 30-35times; 72°C for 10min; 4°C forever), using T7-labelled primers (listed in the Table 3). Illustra GFX PCR DNA, Gel Band Purification Kit (GE Healthcare), or GenElute™ PCR Clean-Up/Gel Extraction Kit (Sigma) was used to purify the PCR amplicons, according to the manufacturer's instructions. The TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific) was used to *in vitro* synthesize dsRNA from purified T7-amplicons as described by the manufacturer. Briefly, transcription reaction was allowed to run for around 16 hrs and was then treated with DNase I. DsRNA was then extracted with phenol:chloroform, precipitated with isopropanol, re-suspended in nuclease-free water, and adjusted to a final concentration of 3μg/μl. Gene silencing efficiency was tested either by immunoblotting if antibodies were available, or by real-time PCR (Table 4).

Table 3. T7-flanked primers used for dsRNA production

Primer	**Primer sequence (5'-3') - The T7 promoter sequence is underlined.**
LacZ-F	TAATACGACTCACTATAGGGAGAATCCGACGGGTTGTTACT
LacZ-R	TAATACGACTCACTATAGGGCACCACGCTCATCGATAATTT
TEP1-F	TAATACGACTCACTATAGGGTTTGTGGGCCTTAAAGCGCTG
TEP1-R	TAATACGACTCACTATAGGGACCACGTAACCGCTCGGTAAG
LRIM1-F	TAATACGACTCACTATAGGGAATATCTATCTCGCGAACAATAA
LRIM1-R	TAATACGACTCACTATAGGGAATATCTATCTCGCGAACAATAA
CLIPA2-F	TAATACGACTCACTATAGGGATCCTAACAACGGCACACTGTGTGA

CLIPA2-R	<u>TAATACGACTCACTATAGGG</u>TCCTGATCGCCATGATTGGTGGTGCT
CLIPA8-F	<u>TAATACGACTCACTATAGGG</u>AACAACGAACCCGTAGAATATG
CLIPA8-R	<u>TAATACGACTCACTATAGGG</u>GGTTAGCGCCTCGATACC
SPCLIP1-F	<u>TAATACGACTCACTATAGGG</u>GTCACCGAACACGTCCAAC
SPCLIP1-R	<u>TAATACGACTCACTATAGGG</u>CTGCATGGCCCTACGTCTA
Rel1-F	<u>TAATACGACTCACTATAGGG</u>AGAATCAACAGCACGACGATGAG
Rel1-R	<u>TAATACGACTCACTATAGGG</u>AGATCGAAAAAGCGCACCTTAAT
Rel2-F	<u>TAATACGACTCACTATAGGG</u>AGACGGAGAAGTCGAAGAAAACG
Rel2-R	<u>TAATACGACTCACTATAGGG</u>AGACACAGGCACACCTGATTGAG
APOIII-F	<u>TAATACGACTCACTATAGGG</u>TCCAGTCGATCATGAGCATCA
APOIII -R	<u>TAATACGACTCACTATAGGG</u>AGCTTCTTGAGCGCGTCCT
Fondue-F	<u>TAATACGACTCACTATAGGG</u>TGCCAGCTCTTCGCACAACAGT
Fondue -R	<u>TAATACGACTCACTATAGGG</u>GCATATTGAACGTCTTGGGTT
Lipophorin-F	<u>TAATACGACTCACTATAGGG</u>CGAACACCAAGGACCAGTCG
Lipophorin-R	<u>TAATACGACTCACTATAGGG</u>TGGTGTTGGTTGGTGCGA
Jun-F	<u>TAATACGACTCACTATAGGG</u>CGGTACAACACGTCCATCAC
Jun-R	<u>TAATACGACTCACTATAGGG</u>AGGGTGACACTGTTGGCACT

Table 4. Efficiency of gene silencing by RNAi

Gene	Knockdown efficiency (± SD)
Rel1	69 ± 3.5
Rel2	48 ± 4
Fondue	69 ± 3
Apo-III	86.5 ± 5
*Jun**	77.8 ± 4

Efficiency of *Jun* silencing was measured in hemocytes of sugar fed mosquitoes at 3 days after dsRNA injection. For the remaining genes, it was measured in sugar fed whole mosquitoes at 3-4 days after dsRNA injection.

2. *RNA extraction, reverse transcription for cDNA synthesis, and real-time PCR*

Around 12-15 mosquitoes per genotype were stored in 300µl TRIzol Reagent (Invitrogen). Nucleic acids were extracted with chloroform (1:5 chloroform to TRIzol ratio) and treated with DNase I. Total RNA was further extracted with phenol/chloroform, precipitated with isopropanol (0.7 volumes) and the collected RNA pellet was re-suspended in nuclease-free water. cDNA was synthesized from 1 µg of RNA by reverse transcription using the iScript™ cDNA Synthesis kit (BioRad) according to the manufacturer's protocol. All reagents and volumes used in reverse transcription are listed in Table 5. The complete reverse transcription reaction was incubated for 5min at 25°C followed by 30 min at 42°C and final incubation for 5min at 85°C, and the produced cDNA templates were used in real-time PCR. SYBR® Green Quantitative RT-PCR Kit was used to carry out the real-time PCR reactions. In brief, the previously prepared cDNA samples were diluted 10x, and in a 96-well microtiter plate 5µl of the diluted cDNA was mixed with 12.5µl SYBR Green *Taq* ReadyMix, 2.5

μl nuclease-free-water, 2.5 μl primer forward and 2.5 μl primer reverse (previously standardized by qRT-PCR) (Table 6). The plate was properly sealed, centrifuged at 1000g, 4°C, for 3mins, and placed in a CFx96 Systems light cycler machine (initial denaturation step: 95°C for 3mins followed by a denaturation step: 95°C for 10s repeated 39 cycles, then by an annealing step: 60°C for 30s). The ribosomal S7 gene was used as an endogenous control gene to normalize the relative mRNA expression level of each tested gene and the qRT-PCR Ct values were used to calculate the relative variation in the mRNA levels. In cases were antibodies were not available, the efficiency of dsRNA silencing was verified using qRT-PCR.

Table 5. Material used in reverse transcription

Component	**Volume/reaction**
5x iScript reaction mix	4 μL
iScript reverse transcriptase	1 μL
Nuclease-free water	Up to 20μL
Total RNA	Equivalent to 1μg
Total	**20 μL**

Table 6. Primers used in real-time PCR

Gene	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**
TEP1	AAAGCTACGAATTTGTTGCGTCA	TTCTCCCACACACCAAACGAA
CLIPA2	GATACTACCTGCACGGGTTGGT	CAGTATAAGGTATCTGCTTCTGATGGC
Cecropin 1	TCATCTTTGTCGTGCTGGCA	TCTTCAGCCGTCCCGCT

Cecropin 3	CGTACAGCCAGTCGATGGTG	AACACATTGCGTCCGAGCTT
Gambicin	ACGCTACTGTGGCTACGGCTA	CGTCTGTCCGTCGCACG
Defensin 1	GCCTTTGTGCCGCTCACT	GCCTTACTGTTGCAGTAACCACC
Fondue	AGCGACACACCCAAAACTCA	GTCGAGGAAGCACTCACTGG
Apolipoprotein III	TGTGCCGTGCAGGGAAGCT	AAGAAGTTGGGCTCCTCGG
Jun	CAGGGCAAGTTTTGAATGCAC	CATTGGGACCGCAAACCAAG
APOIII	TGTGCCGTGCAGGGAAGCT	AAGAAGTTGGGCTCCTCGG
Rel1	CCAACCTCGATCCGGTGTTCA	TAGGTCGGTCGTGGAAAGTGA
Rel2	GCCATTCCGGAAGGTCAAGA	AATGTCCGGATGATGGGCTGA

3. *Generation of HIS-tagged plasmids*

The ligase-independent cloning (LIC) kit (Novagen) was used to clone the entire open reading frames of *CLIPA2* lacking the endogenous signal peptide and stop codon into a *pIEx10* insect cell expression plasmid (Novagen) enclosing a C-terminal 10x HIS-tag according to manufacturer's protocol. The primers used to generate *pIEx10-CLIPA2*^{HIS} are provided below:

Primer	**Primer sequence (5'-3')-Underlined are the extensions of the sequences that allow ligase-independent cloning (Povelones, 2009).**
CLIPA2-F	<u>GACGACGACAAGATG</u>GACTACATCCAACAAGAGCAATG
CLIPA2-R	<u>GAGGAGAAGCCCGGTTT</u>CTCACGCTGTTTGGAGTGTCCC

E. Cell biology

1. *Generation of stable cell lines*

Around $1*10^6$ exponentially growing Sf9 cells were seeded per well in a 6 well-plate. Cellfectin II transfection reagent (Invitrogen) was used to co-transfect the

cells with 2 µg of plasmid *pIEx10-CLIPA2^{HIS}* and 0.2 µg of *pIE1-neo*, according to the manufacturer's instructions. At 24 hours post transfection, cells were gently detached using a cell scraper or a syringe plunger, diluted in Sf-900 medium supplemented with 10% FBS, 50 U/µL penicillin and 50 U/µL streptomycin, (sometimes antibiotic-antimycotic solution:10,000 U/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL Fungizone was also added) and seeded at different densities (2×10^5, 1×10^5, 2.5×10^4, and 1.25×10^4 cells/mL) in a 6-well plate, with a total volume of 4mL/well. Stably secreting cells were selected for approximately 2 weeks by the addition of 1mg/mL G-418 antibiotic in the serum containing medium. Old medium was changed once every five days. After the 2 week selection, selective pressure was gradually dropped to 0.5mg/mL, then to 0.25mg/mL of G-418 antibiotic. Stable cell lines expressing the protein of interest (as determine by western blot) were regularly checked for confluence and maintained always in the presence of 0.25mg/mL of G-418 antibiotic.

F. Antibody Production

1. Production of antisera against CLIPA2

Talon beads (Clonetech) were used to purify recombinant *CLIPA2^{HIS}* proteins from around 40 T75flasks containing around 400-500ml of conditioned media. Briefly, around 500µl talon resins/50ml media were washed 4-5times with 1xPBS, re-suspended in PBS (v: v ratio), and incubated with the conditioned media on a rotator/shaker, at room temperature, for 2-3hours. Beads were then washed 4-5 times with wash solution (1xPBS, 10mM imidazole, pH 7.4), and proteins were eluted with 4 bed volumes of elution buffer (1xPBS, 300mM imidazole, pH 8.0). Bradford assay (Fermentas) was used to quantify the purified recombinant proteins and coomassie staining of SDS-

PAGE gels to check the quality of the protein (i.e. absence of degradation). Approximately 1mg of recombinant CLIPA2 was used to generate rabbit polyclonal antibodies at Eurogentec antibody production facility.

2. *Affinity purification of the CLIPA2 polyclonal antibody*

Polyclonal CLIPA2 produced at the Eurogentec facility was affinity purified as follows. First, an affinity column was prepared by cross-linking around 2-4ml of CLIPA2 protein solution desalted and re-suspended in 1x PBS to the AminoLink Column using Sodium Cyanoborohydride crosslinker with an end-over-end rocking, for 4 hours, at room temperature. The remaining active sites of the column were further blocked by the addition of quenching buffer and Sodium Cyanoborohydride Solution with another 30 minutes of incubation. The column was then washed with at least 10 ml (5 resin-bed volumes) of Wash Solution (1 M NaCl, 0.05% NaN_3). Around 2-3ml of the positive immune serum containing the CLIPA2 antibody was then added to the AminoLink column (Pierce) and the serum mixed resin bed was incubated at room temperature with gentle mixing for 1-2h to allow covalent binding. The column was then washed with 12 ml of Binding/Wash Buffer and the bound proteins were eluted with 8 ml of Elution Buffer (0.1-0.2 M glycine•HCl, pH 2.5-3.0). The pH of each fraction was neutralized by adding 50 µl of Neutralization Buffer per 1 ml of collected eluate. The affinity column was regenerated after elution by washing it with 16 ml of Binding/Wash Buffer to remove any residual protein and reactivate the resin, and was stored upright at 4°C in 2 ml of Binding/Wash Buffer containing 0.05% sodium azide.

G. Protein biochemistry

1. Mosquito hemolymph extraction

Proboscis clipping was used to extract hemolymph proteins from mosquitoes either into 1x non-reducing Laemmli Sample Buffer (BioRad), or into 1x PBS-containing EDTA-free Protease inhibitor cocktail (Roche) when quantification is needed.

2. Western blotting

Hemolymph protein extracts were separated on 8-10% SDS-PAGE and then wet transferred to Immun-Blot PVDF membrane (BioRad). To prevent non-specific antibody binding, membranes were blocked in 3-5% skimmed milk prepared in 1x PBS containing 0.05% Tween 20 (PBS-T) for 1hour at room temperature, then incubated with the primary antibody of interest over night, at 4°C. α- SRPN and α- PPO6 antibodies were used as loading controls. A detailed description of the different antibodies used and their dilutions is provided in Table 7. Membranes were then washed with PBS-T 3times (10min each wash), probed with either horse raddish peroxidase-conjugated α-mouse (1:6000) or α-rabbit secondary antibodies (1:12000) for 1hour at room temperature, and washed again 3x with PBS-T (10min each wash). Bands were revealed by incubating the membranes in ClarityTM Western ECL Substrate (BioRad) for 30s-1min, followed by exposing them to Kodak X-ray films between 1 and 20min depending on the antibody used. Films were developed manually.

Table 7. Primary antibodies used in western blot analysis

Antibody	Type	Species	Dilution	Incubation
α-TEP1*	Polyclonal*	Rabbit	1:1000	1 hr at RT to O.N. at $4^{o}C$
α-CLIPA2**	Polyclonal	Mouse	1:1000	O.N. at $4^{o}C$
α-CLIPA2*	Polyclonal	Rabbit	1:1000	O.N. at $4^{o}C$
α-CLIPA8	Monoclonal	Mouse	1:30	O.N. at $4^{o}C$
α-LRIM1	Polyclonal	Rabbit	1:2000	1 hr at RT to O.N. at $4^{o}C$
α-APOII**	Monoclonal	Mouse	1:100	O.N. at $4^{o}C$
α-SPCLIP1*	Polyclonal*	Rabbit	1:2000	1 hr at RT to O.N. at $4^{o}C$
α-PPO6	Polyclonal	Rabbit	1:2000	1 hr at RT to O.N. at $4^{o}C$
α-SRPN3	Polyclonal	Rabbit	1:1000	1 hr at RT to O.N. at $4^{o}C$

* Affinity-purified antibody
**Melon-gel purified antibody

3. Phenoloxidase enzymatic assay

LacZ, *TEP1*, *CLIPA8*, and *CLIPA2*kd mosquitoes were either injected with *E.coli* bacteria (O.D. 0.4) or sprayed with 10 x 10^{7}spores/ml of WT *B. bassiana* (strain 80.2) fungal suspension. Hemolymph was extracted 3 hours post bacterial infection and 24, 48, and 72h post fungal infection in ice-cold phosphate buffered saline (PBS) containing EDTA-free Protease inhibitor cocktail (Roche). Bradford assay (Fermentas) was used to quantify proteins. Prophenol oxidase enzymatic assay was done as previously described (Schnitger, *et al.,* 2007). Briefly, around 3-5µg of proteins were

used per reaction. After the addition of the L-DOPA substrate, absorbance was measured at 490nm, at a 10min interval, for a period of 1 hour using a Multiskan Ex microplate reader (ThermoLabsystems).

4. Immunoprecipitations

a. CLIPA2 immunoprecipitation

For small-scale immunoprecipitation (IP) experiments, around 5µg of affinity purified CLIPA2 antibody was covalently cross-linked to 20-40µl AminoLink Plus Coupling Resin using the Pierce Co-IP kit (Thermo Scientific) as described by manufacturer. Hemolymph was extracted from around 100 *B. bassiana* infected mosquitoes per IP reaction directly into 200 µl ice-cold IP buffer containing 1xPBS, 0.01% TritonX-100, and EDTA-free Protease inhibitor cocktail. Hemolymph extracts were incubated with the antibody-coupled beads on a rotating wheel at 4°C, overnight. Next day, the unbound fraction (flow through) was collected and supplemented with 1x non-reducing Laemmli Sample Buffer (BioRad). Beads were washed 5-6 times with the IP buffer, and bound proteins were eluted with 100 µl elution buffer (0.1-0.2 M glycine•HCl, pH 2.5). The eluent was concentrated down to 50 µl using centrifugal filter units with 10KDa cutoff (Amicon ® Ultra). Unbound and bound protein samples were analyzed by western blot as previously described.

For large-scale immunoprecipitation experiments, hemolymph was extracted from around 800-1000 *B. bassiana* infected mosquitoes 72 hours post infection. Co-IP was performed exactly as described for small scale IP. The eluents were then precipitated with trichloroacetic acid and sent to the proteomic platform at IGBMC (Strasbourg) for protein identification using mass spectrometry. In brief, protein samples were reduced, alkylated and digested with LysC and trypsin at 37°C overnight.

An Ultimate 3000 nano-RSLC (Thermo Scientific) coupled in line with an Orbitrap ELITE (Thermo Scientific) was used to analyze the samples. A C18 nano-column containing a linear gradient of acetonitrile was used to separate the peptides. Data was analyzed by means of a Top 20 Collision Induced Dissociation (CID) data-dependent mass spectrometry, and processed by database searching using SequestHT (Thermo Fisher Scientific) with Proteome Discoverer 1.4 software (Thermo Fisher Scientific) against an *Anopheles gambiae* database (Swissprot and TrEMBL together, release 2015_01, 18304 entries). Precursor and fragment mass tolerance were set at 7 ppm and 0.5 Da respectively. Trypsin was set as enzyme, and up to 2 missed cleavages were allowed. Oxidation (M) was set as variable modification, and Carbamidomethylation (C) as fixed modification. Peptides were filtered with a score versus charge state (1.5 z_1, 2.5 z_2, 3 z_3 and 3.2 $z_{\geq 4}$) and rank 1. Proteins were identified with at least 2 different peptides.

H. Immunohistochemistry and microscopy

To determine the interaction of immune proteins with fungal surfaces, around 200 wild type or GFP-expressing *B.bassiana* (242 GFP strain) spores were injected into individual female mosquitoes. Abdomens were dissected at different time points, and fixed in 4% formaldehyde for 50 minutes, then washed 3 times with 1xPBS (10minutes each wash), and blocked with 1xPBS containing 1-2% BSA and 0.05%TritonX-100 (PBT) for 1h at room temperature. Abdomens were then incubated with primary antibodies (Table 8) overnight, at 4°C, and the following day, were washed again three times with PBT (10minutes each wash) and incubated with Alexa-546 (1:800), 488 (1:600), or 647 (1:800) conjugated secondary α-rabbit or with Alexa-633 conjugated α-mouse (1:800) at room temperature, for 1-2 hours, washed with 3 times with PBT

(10minutes each wash), incubated in PBS containing Hoechst stain (1:10000), then mounted in ProLong® Gold antifade reagent (Invitrogen). Zeiss LSM 710 META confocal microscope was used to collect the fluorescent images.

To study the localization of immune proteins to bacterial surfaces, we used two approaches: fluorescent microscopy and bioparticle surface extraction assay. In the first approach, we conducted a bioparticle surface extraction assay as previously described in page 144 of this thesis. In the second approach, GFP-expressing-Ampicilin resistant *E. coli* culture were grown overnight at 37°C. Bacterial were pelleted by centrifugation, and washed four-five times with 1xPBS. The bacterial pellet was re-suspended in 1xPBS, and the $O.D._{600nm}$ of the bacterial suspension was measured and adjusted to 0.8. Individual female mosquitoes were then injected with 69 nl of the bacterial suspension. Half an hour post *E.coli* injection, hemolymph was extracted from around 25-30 mosquitoes into 5 µl of 15mM Tris-Hcl buffer (pH 8.0) containing EDTA-free Protease inhibitor cocktail -Roche), and deposited on a SuperFrost adherent slide (Thermo scientific). The region where the drop was loaded was marked. The slide was left at room temperature to air-dry and to allow hemocytes and bacteria to adhere to the slide. After drying, adhered cells were fixed for ten minutes, at room temperature, in 4% formaldehyde. The slides were then washed three times with 1x PBS (10 minutes each wash), blocked in PBS containing 2% BSA, for one hour at room temperature, then incubated with the primary antibody overnight at 4°C (Table 8). Cover slips were added to the slides which were then placed in a humid chamber to avoid drying of the primary antibody solution. Next day, the slides were washed 3-4 times with 1xPBS, and incubated with Alexa-546 (1:800), 488 (1:600), or 647(1:800) conjugated secondary α-rabbit or with Alexa-633 conjugated α-mouse (1:800) at room temperature, for 1-2 hours. The slides were washed again with 1xPBS, stained with Hoechst (1:10000)

prepared in 1xPBS, and mounted in ProLong® Gold antifade reagent (Invitrogen). Zeiss LSM 710 META confocal microscope was used to collect the fluorescent images.

Table 8. A list of primary antibodies used in immunohistochemistry

Antibody	Type	Species	Dilution	Incubation
α-TEP1	Polyclonal*	Rabbit	1:350	O.N. at 4°C
α-CLIPA2	Polyclonal*	Rabbit	1:350	O.N. at 4°C
α -CLIPA8	Polyclonal*	Rabbit	1:350	O.N. at 4°C
α-PPO6	Polyclonal	Rabbit	1:500	O.N. at 4°C
α-APO I	Monoclonal	Mouse	1:50	O.N. at 4°C
α-APO II**	Monoclonal	Mouse	1:50	O.N. at 4°C

* Affinity-purified antibody
**Melon gel-purified antibody

I. Mosquito fecundity assay

69 nl of an *E. coli* culture re-suspended in 1xPBS and adjusted to $O.D._{600}$ 0.4 was injected in *LacZkd* and *CLIPA2kd* female mosquitoes. PBS injected wild-type naive female *Anopheles* and non-injected ones were used as controls. Four days post infection; mosquitoes were given a blood meal by allowing them to feed on the same naive mouse. Individual blood-fed female mosquito was then placed in a paper cup, and the eggs laid were counted 48h post blood feeding using a stereoscope. From 2 independent biological repeats, a total of at least 72 individual female *Anopheles* per experimental group was used.

To examine the effect of fungal infection on mosquito fecundity, female *Anopheles* were sprayed with 10×10^7 conidia/ml of wild type or Bb-Aa1 strain fungal suspension. Twenty four hours post infection; mosquitoes were given a blood meal. Individual blood fed female mosquito was then placed in a paper cup, and the laid eggs were counted two days post blood feeding. The variability between one mosquito and the other was reduced by collecting all females from the same batch, and having the same age. The Mann-Whitney test was used to perform the statistical analysis, and medians were considered significant if $p < 0.05$.

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